CONTENTS

May–June, 1961. Vol. 40, No. 3

391 Editorial Views and News

392 Announcement of 1962 General Meeting of the International Association for Dental Research

393 Effect of Fluoride Ingestion on the Size of the Cusp of Carabelli during Tooth Formation—GERALD J. COX, SIDNEY B. FINN, and DAVID B. AST

396 Personality Correlates of Periodontal Disease—ERNEST G. BAKER, G. HAMILTON CROOK, and ELSBETH D. SCHWABACHER

404 Cutting Studies at Air-Turbine Speeds—J. O. SEMMELMAN, P. R. KULP, and L. KURLANSIK

411 Thermal Conductivity of Tooth Structure, Dental Cements, and Amalgam—R. G. CRAIG and F. A. PEYTON

419 A Study of Posterior Mandibular Movements from Intercuspal Occlusal Position—WILLIAM L. KYDD and ALLAN SANDER

426 Experimental Carcinogenesis in Submandibular Glands of Hamsters—A. P. CHAUDHRY, D. H. REYNOLDS, R. J. GORLIN, and R. A. VICKERS

433 Amount, pH, Buffer Effect, and Amylolytic Activity of Parotid Saliva between and Immediately Following Meals—BO KRASSE

436 Histochemistry of Oral Mucous Membrane: Total Protein, Sulfhydryls, Disulfides, Ribonucleic Acid, and Desoxyribonucleic Acid—LOUIS A. CANCELLARO, JULES KLINGSBERG, and EARL O. BUTCHER

446 Studies on the Antibacterial Action of Human Saliva. II. Observations on the Mode of Action of a Lactobacillus Bactericidin—BERNARD J. ZELDOW

454 Studies on the Amino Acid Decarboxylases of Oral Lactobacilli—WILLIAM R. BIBB and W. R. STRAUGHN

461 The Comparative Distribution of Glycogen, Succinic Dehydrogenase, and Acid Mucopolysaccharides in Different-aged Animals—JULES KLINGSBERG, LOUIS A. CANCELLARO, and EARL O. BUTCHER

470 Standardized Paper Electrophoresis of Parotid Saliva: A Study of Variables in Technique and Flow Rate as Factors in Resolution—SHELDON HOLEN, NORMAN HABER, and IRMA TUCK-WEISS

479 Whole Saliva, Salivary Sediment, and Supernatant as Fermenting Agents for Foods—DONALD J. BECK and BASIL G. BIBBY

486 Acid Production during the Fermentation of Starches by Saliva—DONALD J. BECK and BASIL G. BIBBY

492 A Comparison of Two Types of Clinical Examinations: The Effect on Observed Caries Prevalence and Increments—P. R. Weisenstein, John K. Peterson, and Paul E. Norris

497 Evaluation of Techniques for Analyzing Cavity Design for Amalgam Restoration—David B. Mahler, Louis G. Terkla, and Leonard N. Johnson

504 Further Studies on the Presence of Se^{75} in Rat Saliva and Teeth after Intracardiac Injection of Radioactive Sodium Selenite—C. K. Claycomb, F. M. Sorenson, D. C. Gatewood, E. B. Jump, and M. E. Weaver

511 The Influence of Antibiotics on the Periodontal Syndrome in the Rice Rat—James H. Shaw, Derrick Griffiths, and Aina M. Auskaps

520 Changes in the Connective Tissue of Rats Fed Toxic Diets Containing Molybdenum Salts—C. A. Ostrom, R. Van Reen, and C. W. Miller

529 Additional Studies on Fluoride Uptake by Enamel from Certain Dental Materials—Richard D. Norman, James R. Platt, Ralph W. Phillips, and Marjorie L. Swartz

538 Eruption of Irradiation-produced Rootless Teeth in Monkeys—Joseph M. Gowgiel

548 Pre-eruptive Factors of Tooth Rotation and Axial Inclination—Young H. Kim, F. R. Shiere, and H. R. Fogels

558 The Caries-Conduciveness of a High-Sucrose Diet with and without the Addition of Brewer's Yeast—John Haldi, Winfrey Wynn, and Mary L. Law

561 Dissolution Rates of Enamel and Dentin in Acid Buffers—Michael G. Buonocore

571 Phosphatase in Micro-organisms Cultured from Carious Dentin and Calculus—W. H. Bowen

578 Effect of Organic Ions on Solubility of Enamel and Dentin in Acid Buffers—Theodore A. Koulourides and Michael G. Buonocore

594 Dental Caries and Nutrition in Eskimo Scouts of the Alaska National Guard—A. L. Russell, C. Frank Consolazio, and Carl L. White

604 Periodontal Disease and Nutrition in Eskimo Scouts of the Alaska National Guard—A. L. Russell, C. Frank Consolazio, and Carl L. White

614 Relation of Protein, Carbohydrate, and Fat Intake to the Periodontal Syndrome—James H. Shaw and Derrick Griffiths

RESEARCH ANNOTATIONS

622 Natural or Derived Materials Which Replace Ascitic Fluid for the Growth of an Oral Spirochete—T. A. Nevin and H. V. Jordan

623 Toxicity Study of Stannous Fluoride in Swiss Strain Mice—V. A. Segreto, R. A. Yeary, R. Brooks, and N. O. Harris

624 Cytology and Morphogenesis of *Entamoeba Gingivalis*—Wayne W. Wantland, Edna M. Wantland, and John W. Remo

625 The Nasal Septum: An Endochondral Growth Center—Louis J. Baume

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

627 Sections and Officers

628 Supporting Associates

Editorial Views and News

Dentistry constitutes a young area in the health sciences and thus does not have a long list of great personalities, as do some of the older sciences. It became very apparent, however, during the course of the banquet meeting of the International Association for Dental Research in Boston that we were honoring a man who had contributed greatly to the field of dentistry though not actually a dentist. (The proceedings and abstracts of that same meeting, held March 23–26, 1961, will be published in the July–August issue of the *Journal of Dental Research.*)

The Dr. Souder Bronze Plaque.—George M. Hollenbach, himself an outstanding figure in dentistry, had this to say about Wilmer Souder upon presenting to the Dental Division of the National Bureau of Standards a bronze plaque of Dr. Souder: "It has been my belief that those who have made worthwhile contributions to the science and art of dentistry should be given due recognition for their efforts.

"It is my pleasure . . . to so recognize a man who is second to none from the standpoint of his contributions to dentistry. This man is Wilmer Souder, A.B., M.S., Indiana University, Ph.D. *magna cum laude,* University of Chicago, in physics and chemistry.

"After receiving his doctorate, Dr. Souder remained at the University of Chicago and acted first as assistant to Dr. A. A. Michelson, who was head of the Physics Department at the University. Upon his retirement Dr. Robert A. Millikan succeeded Michelson. Dr. Souder also acted as assistant to Dr. Millikan. Both Michelson and Millikan were recipients of the Nobel Prize for physics.

"With this very liberal scholastic background Dr. Souder is, therefore, well fitted for research in the physics and chemistry of dental materials.

"Dr. Souder was the founder in 1919 of dental research at the National Bureau of Standards. It was through his persistent efforts that the American Dental Association became interested in 1928 and, at that time, became a vital part of the research on dental materials at the Bureau. Dr. Souder became Chief of the Division of Metrology in 1945.

"We all know dentistry has had standards for drugs since the development of the early pharmacopeias at the beginning of the nineteenth century, but it was not until 1920 that the first standard was formulated for a dental material.

"The first specification—that for dental amalgam—was the handiwork of Dr. Souder and his co-workers at the National Bureau of Standards. With the aid of this specification, an operative dentist using amalgam could be assured that the basic material was adequate. It then became encumbent upon him to use amalgam in the best possible manner. There is considerable evidence that the profession has met this challenge adequately, and thus the quality of restorative dentistry in our country has been greatly improved.

"It is not only my belief but, I am sure, the belief of all of you present that the benefits that our profession has derived from the dental research of the Bureau are beyond calculation. Not only have specifications been set up for many other materials but, in addition to this, extensive studies have been made on them. This work, which now constitutes almost four hundred reports, has had a pronounced effect on the practice of dentistry, on the manufacture of dental materials, and on dental education. May it long continue to make the dental profession research-conscious.

"During my entire professional career I have been greatly interested in the problem of precision casting. I have expended a great deal of time and effort in this study. However, if it had not been for one of Dr. Souder's contributions, all my efforts would have been of little avail. This contribution, which is so commonplace today that we think little about it, was the use of the asbestos liner in a casting flask. This permitted the investment to undergo its natural cycle of expansion. Studies recently made have shown clearly that the liner functions in other capacities than this. If we were denied the use of this one simple technical step, the excellence of our product in the realm of casting could not possibly be maintained at its present high level.

"At the beginning of this . . . address I stated that all those who have made outstanding contributions to the dental profession should receive recognition for what they have done. In the case of certain individuals who have made exceptionally valuable contributions it is my feeling that, in addition to recognition, they should be memorialized.

"It is now my pleasure as the donor to present, on behalf of the Dental Materials Group of the International Association for Dental Research, to the National Bureau of Standards a bronze plaque of Dr. Souder, together with an inscription which I will now read: 'Dr. Wilmer Souder, Founder of Dental Research at the National Bureau of Standards, Washington, D.C., in 1919.' "

—F.J.O.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Fortieth Annual Meeting, March 15, 16, 17, and 18, 1962
St. Louis, Missouri

This meeting will convene in the Jefferson Hotel on the third Thursday, Friday, Saturday, and Sunday of the month of March followed by the meeting of the American Association of Dental Schools. The I.A.D.R. met in St. Louis at the same site in 1956. A previous St. Louis meeting was held in 1941.

Effect of Fluoride Ingestion on the Size of the Cusp of Carabelli during Tooth Formation

GERALD J. COX, SIDNEY B. FINN, and DAVID B. AST

Research Department, School of Dentistry, University of Pittsburgh, Pittsburgh, Pennsylvania; School of Dentistry and Medical College of Alabama, Birmingham, Alabama; and Bureau of Dental Health, Department of Health, State of New York Albany, New York

The cusps of Carabelli, on the upper molar teeth of man, are variable in occurrence, as exemplified by Dietz,[1] who found them present on first molar teeth in 72.3 per cent of 747 casts from Army personnel. Dietz[1] also found them to be generally present on both teeth and in approximately the same size. Meredith and Hixon[2] have succinctly reviewed the literature on size, frequency, and bilaterality of the cusp. For their studies of 100 casts, they had agreement in placement in four categories of size for 73 sets, with a discrepancy in two categories for only one set. They found cusps to be present in 84 of the casts.

As to morphology of teeth related to nutrition during formation of teeth, Cox and Levin[3] found a higher rate of fusion of minor cusps to form major cusps in rats from mothers on a synthetic ration than from mothers on a 100 per cent meat diet. Cox and Hagan[4] found higher rates of occurrence of pits in second human deciduous molar teeth than in first molars and surmised, on the basis of the above observations of Cox and Levin,[3] that this was the result of better nutrition during the formation of the first molars.

Norvold and Armstrong[5] provided water with 20 p.p.m. F for rats during pregnancy and lactation, and 0.1 mg. fluorine was given daily to each suckling rat for the first 21 days of life. In 11 of the 26 rats of this group, "the mesio-buccal cusp of the upper third molar was missing. In none of the other 165 animals employed in this study was this cusp found to be absent."

Paynter and Grainger[6] found smaller upper first molar teeth in rats born to and nursed by mothers on rations deficient in vitamin A or with abnormally high phosphate content or when their water contained 12 p.p.m. sodium fluoride. They found the smaller size of the teeth from vitamin A deficiency to be related to the smaller size of these animals, but the high phosphate or fluoride rations were directly causative of smaller size. As to the form of the teeth, the depth of the mesial fissures of the tooth was less, and the angulation of the sides of this fissure was greater in the young from mothers maintained on the high-phosphate diet for 10 weeks prior to mating.

One of us (G. J. C.) has failed to find the cusp of Carabelli in four persons with severe mottled enamel, and its absence has been reported in two other cases.[7] This,

---

This investigation was supported by U.S.P.H.S. research grant D-1007 from the National Institute of Dental Research, National Institutes of Health.

Received for publication October 13, 1959.

with the above observations of Norvold and Armstrong,[5] suggested that a diminished frequency of the cusp would be found in first maxillary permanent molar teeth formed by use of water with fluoride at 1 p.p.m. level, compared with teeth from a non-fluoridated area.

MATERIALS AND METHODS

From children of Kingston and Newburgh, New York, 764 casts were available for study of the frequency of occurrence of the cusp of Carabelli on the first maxillary molar tooth of the permanent dentition. The casts were from children aged six to ten in Newburgh and Kingston. The Newburgh children had had 7 years of fluoride ex-

TABLE 1

DISTRIBUTION OF SIZES OF CUSP OF CARABELLI IN CHILDREN FROM KINGSTON AND NEWBURGH

	Size								Total
	a	b	c	d	e	f	g	h	
Kingston:									
Number....	25	40	44	50	60	57	29	13	318
Per cent....	7.9	12.6	13.8	15.7	18.9	17.9	9.1	4.1	100.0
Newburgh:									
Number....	28	21	41	63	63	82	40	13	351
Per cent....	8.0	6.0	11.7	17.9	17.9	23.4	11.4	3.7	100.0

TABLE 2

DISTRIBUTION OF THE FREQUENCY OF TWO SIZES OF CUSP OF CARABELLI BETWEEN KINGSTON AND NEWBURGH

Classes	Kingston	Newburgh	Totals
a, b, c, d.....	159	153	312
e, f, g, h.....	159	198	357
Total.....	318	351	669

perience, at 1.0 p.p.m. F, beginning May 2, 1945, whereas the Kingston children used a fluoride-deficient water supply.[8]

Most of the casts were coded and had been stored in random fashion. They were placed by one of us (S. B. F.) in eight categories of size of the cusp,* ranging from no discernible cusp, designated as "a," to the largest size, "h" (Table 1). The last four categories were divided from the mesial-lingual cusp by a definite and distinguishable groove. Of the 669 casts with the first molars present, 318 were from Kingston and 351 from Newburgh. The data of distribution are shown in Table 1.

In the course of study, the opinion was expressed that classes a, b, c, and d could well be combined, excepting possibly a, and the e, f, g, and h categories were also combined. This action is shown in Table 2.

* Casts supplied to us by Dr. A. A. Dahlberg, presenting eight sizes, served as standards.

RESULTS

The χ^2 test of this 2×2 table yields 2.75, or a probability of about 0.10 that fluoride at the level of 1.0 p.p.m. causes an increase in the size of the cusp of Carabelli.[8]

Fluoride present in a community water supply at the level of one part per million does not affect the size of the cusp of Carabelli.

REFERENCES

1. Dietz, V. H. A Common Dental Morphotropic Factor: The Carabelli Cusp, *J.A.D.A.*, **31**:784, 1944.
2. Meredith, H. V., and Hixon, E. H. Frequency, Size, and Bilateralism of Carabelli's Tubercle, *J. D. Res.*, **33**:435, 1954.
3. Cox, G. J., and Levin, M. M. Alteration of Tooth Form in the Rat by the Preeruptive Diet, *J. D. Res.*, **23**:191, 1944.
4. Cox, G. J., and Hagan, C. W. The Relative Occurrence of Pits in Human Deciduous Molars and the Possible Relationship to Nutrition, *J. D. Res.*, **28**:646, 1949.
5. Norvold, R. W., and Armstrong, W. D. Mechanism of Fluorine Inhibition of Caries in the Rat, *J. D. Res.*, **22**:243, 1943.
6. Paynter, K. J., and Grainger, R. M. Relation of Nutrition to the Morphology and Size of Rat Molar Teeth, *J. Canad. D. A.*, **22**:519, 1956.
7. Personal communication from Dr. Leo J. Wirth.
8. Ast, D. B. The Newburgh-Kingston Caries Fluorine Study. VII. Correlation of Ingested Water Fluorides to Dentofacial Development. A Preliminary Report, *Am. J. Orthodontics*, **41**:45, 1955.
9. Fisher, R. A., and Yates, F. *Statistical Tables for Biological Agricultural and Medical Research*, pp. 3, 27. London: Oliver & Boyd, 1938.

Personality Correlates of Periodontal Disease

ERNEST G. BAKER, G. HAMILTON CROOK, and ELSBETH D. SCHWABACHER
School of Dentistry, University of California

Results of a recently reported experience in clinical practice regarding personality factors in dental disease prompted the present study. The etiology of periodontal disease is complicated, and many processes are at work. Today it is widely felt that psychological factors enter into this disease process.

The accompanying bibliography will illustrate the rather extensive literature on the subject and the general transition from merely handling the nervous person to regarding the psychological influence on the disease process itself. Possible influence of emotions on the etiology of dental disorders is the subject of many articles, particularly by Campbell,[1] Ryan,[2] Burstone,[3] and Landa.[4] More recent publications by Miller,[5] Jones,[6] Rosenberg,[7] Roth and Stone,[8] Bell,[9] and Glickman[10] cite psychic disturbances as possible etiologic factors in oral disease and stress the need for evaluating their significance. Campbell,[1] Landa,[4] and Moulton[11] draw the dentist's attention to the aid that can be gained in diagnosis and treatment by detailed personality studies and suggest use of a psychologically skilled person for this purpose. Thompson,[12] Walsh,[13] Zeifert,[14] Balters,[15] Raginsky,[16] Zaidens,[17] and Weiss[18] are among those who point out that the oral zone is especially vulnerable to psychosomatic disturbances because of its importance in the psychosexual development of the individual, the mouth being the organ of the infant's first awareness, its satisfactions, frustrations, and hostilities. Some of these authors stress that the dentist needs to recognize the difference in personalities, with their attendant physiological disturbances, that can be manifested in the mouth region.

Results of investigations by Manhold and Manhold,[19] Manhold,[20] Manhold and Rosenberg,[21] and Manhold and Jones[22] indicate that a statistically significant correlation could be established between neurotic tendencies and certain personality variables with periodontal disease and that opposite personality traits could be tentatively correlated with a high incidence of caries. Studies conducted by Goldberg, Ambinder, Cooper, and Abrams[23] and Moulton, Ewen, and Thieman[24] reveal that specific emotional stress and acute anxiety states can be correlated with acute inflammatory processes in the mouth, while Moulton[11] also demonstrates conflicts of longer standing in chronic cases.

In the review of the present literature the authors were struck with the fact that there is an urgency for proper evaluation in this important field. As knowledge grows concerning psychosomatic medicine and dentistry, it becomes imperative that studies be undertaken to clarify these impressions and relationships.

This study was made possible in part by the Jesse F. Matteson Fund in the School of Dentistry, University of California.

Received for publication March 9, 1960.

EXPERIMENTAL METHODS

A total number of 102 subjects was used in this investigation. Forty normals, 26 psychotics and 36 psychoneurotics were included. Normals were defined as those who had never had, or were not receiving, psychotherapy and had no apparent need for it. These were employees of Langley Porter Neuropsychiatric Institute, a few students, and several doctors. The psychotic and psychoneurotic patients had been diagnosed by the staff of the Langley Porter Neuropsychiatric Institute and were under treatment there at the time of the study.

The dental part of the study was actively pursued by one of the authors as a representative of the Division of Periodontology. He examined all the subjects from the standpoint of oral disease, particularly directed toward periodontal manifestations. These subjects were sent to him without his knowledge of their emotional status or

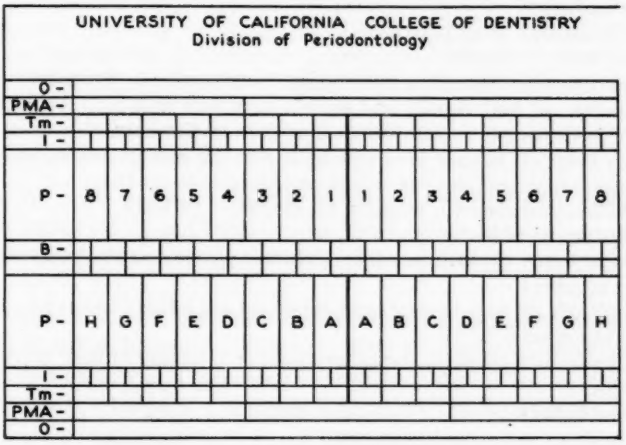

UNIVERSITY OF CALIFORNIA COLLEGE OF DENTISTRY
Division of Periodontology
O -
PMA -
Tm -
I -
P - 8 7 6 5 4 3 2 1 1 2 3 4 5 6 7 8
B -
P - H G F E D C B A A B C D E F G H
I -
Tm -
PMA -
O -

FIG. 1.—Chart used to record amount of bone resorption

diagnosis. The oral examination was processed according to the following plan. A full-mouth set of roentgenograms was taken at the same time that the oral examination was made, and these were later evaluated. The amount of bone resorption was evaluated by a careful examination of the X-rays and recorded on a chart (Fig. 1). The resorption was given a severity rating 0-1-3 or 5 and designated "B" on the chart.

Absence of bone resorption (0) was taken to mean primarily an intact and demonstrable lamina dura with the alveolar crests in a reasonably normal position for the age of the patient. Mild resorption (1) was taken to indicate a definite break in the lamina dura, with resorption extending within the limits of the coronal third of the tooth. Moderate resorption (3) was that which involved a third of the tooth root and more, with its limits at half the coronal roots. Severe resorption (5) was that which extended below the coronal half of the tooth to or including the apical third and involving the bifurcations and/or trifurcations.

The second category used is indicated by the abbreviation "P" and is the actual

measurement of the pockets in millimeters on the mesial and distal root surfaces of every tooth.

The above criteria[25] were the ones used in this present paper. However, the severity 0-1-3-5 of the occlusion lettered *O* on the chart; irritation, letter *I;* inflammation, *PMA;* tooth mobility, listing, and migration, *TM,* were also evaluated and charted.

In the investigation of the relationships between periodontal disease and personality factors, two methods were used to obtain personality data. First, interviews with each subject were conducted to obtain information concerning the variables in the hypothesized personality profile. The interviewer knew nothing about the periodontal condition of the subjects. The interview schedule permitted quick recording of this information, and ratings were made from these records.

A list of the personality variables rated, together with a number of objective and personal history items which were also rated, follows:

I. Objective variables
   1. *Age* (by decades, from 20 to 69)
   2. *Sex* (0, male; 1, female)
   3. *Socio-economic status* (0, comfortable; 1, marginal; 2, dependent)
   4. *Nationality* (0, both subject and parents were born in U.S.: 1, subject born in U.S.; one or both parents foreign-born; 2, subject foreign-born)
   5. *Broken home* (0, subject lived with both own real parents continuously until age 16; 1, subject separated from one or both parents prior to age 16)
   6. *Psychiatric diagnosis* (0, normal; 1, psychiatric patient)

II. Personal history variables
   7. *Illness in family* (0, little or none; 1, chronic physical or mental illness in immediate family member)
   8. *Illness in self* (0, none beyond usual childhood and minor illnesses; 1, chronic physical illness in subject)
   9. *Food habits* as a child (0, normal or average; 1, history of poor nutrition or feeding difficulties)
   10. *Personal habits*
      a) Smoking (0–3, none to extreme)
      b) Drinking (0–3, none to extreme)
      c) Drugs (0–3, none to extreme)
   11. *Neurotic traits of childhood*
      a) History of *thumbsucking* (0, absent; 1, present)
      b) History of *nailbiting* (0, absent; 1, present)
      c) History of *tooth-grinding* (0, absent; 1, present)
   12. *Family adjustment* as a child (to parents and siblings)
      General (0–4, excellent to very poor)
   13. *Feelings of acceptance-rejection* as a child, by family (0, subject felt completely accepted; 1, intermediate or mixed feelings; 2, subject felt rejected)
   14. *Feelings of independence-dependence* upon family, as a child (0, independent; 1, intermediate or mixed feelings; 2, dependent)
   15. Feelings of *friction with family members* as a child (0, rarely or never; 1, sometimes; 2, very often)

III. Personality variables (current)
   16. *Social adjustment,* general (0–4, excellent to very poor)
   17. Feeling of *identification with group* (0, strong identification; 1, moderate identification; 2, little or no group identification)
   18. *Dominance-submissiveness* in social relationships (0, dominant; 1, neither or mixed; 2, submissive)

19. Feelings of *adequacy-inadequacy* relative to others (0, no feeling of inadequacy; 1, moderate feelings of inadequacy; 2, extreme feelings of inadequacy)
20. *Hostility expressed directly* to others (0, little or none; 1, moderate or appropriate amount; 2, excessive amount)
21. *Independence-dependence,* general (0–4, complete independence to extreme dependence
22. *Marital adjustment* (to present marriage, or, if presently unmarried, to previous marriage if any) (0–4, very happy to very unhappy)
23. *Occupational adjustment,* general (0–4, excellent to very poor)
24. *Drive toward achievement* (0, strong; 1, moderate; 2, weak)
25. *Occupational stability* (0, very stable; 1, intermediate; 2, unstable)
26. *Intrapersonal adjustment,* general (self-adjustment) (0–4, excellent to very poor)
27. *Anxiety, guilt* (0, little or none; 1, moderate; 2, excessive)
28. *Repressiveness* (0, little; 1, moderate; 2, excessive)
29. *Rigidity* (0, little or none; 1, moderate; 2, excessive)
30. *Hostility, over-all amount* (0, little; 1, moderate; 2, excessive)
31. *Conflict,* conscious and unconscious (0, little; 1, moderate; 2, excessive)
32. *Somatization* (tendency for subject to develop psychogenic physical complaints or psychosomatic disorders) (0, absent; 1, present)
33. *Lack of energy* (0, a great deal of energy; 1, a moderate amount of energy; 2, very little energy)
34. *Tension* (0, little or none; 1, moderate; 2, excessive)
35. *General, over-all adjustment* (0–4, excellent to very poor)

No attempt was made to insure that the personality variables selected would all be independent of one another, and some overlap among them is to be expected. There is reason to believe from their variety that several dimensions of personality are tapped by the ratings.

The other method of obtaining personality information employed the Minnesota Multiphasic Personality Inventory (MMPI). This is a well-validated personality inventory designed to serve as a psychiatric diagnostic aid; it scores on a number of scales, each of which identifies a common psychiatric disorder or tendency thereto. There are several validating scales to throw light on the probable accuracy with which the diagnostic scales represent the true situation for the subject. The diagnostic scales are as follows: hypochondriasis, depression, hysteria (conversion), psychopathic deviate, masculinity-femininity, paranoid reaction, psychasthenic (obsessive-compulsive-reactions), schizophrenia, hypomania.

The separate measures of periodontal disease utilized have been described above. The principal measure used for comparison with personality data is a compound one, representing a combination of two measures: (1) the average amount of bone recession per septum as estimated from X-rays and (2) the average depth of pockets per septum. This was accomplished by first normalizing the distributions, then equating them in mean and standard deviation. The two measures were then added together. The resulting distribution was divided into five intervals on the basis of its own standard deviation, and index scores of 0–4 assigned. An individual score of 0 on this scale represents little or no periodontal disease, a score of 2 represents an average amount (for our sample), and a score of 4 indicates severe involvement.

On personality variables for which the scores are distributed into three or more intervals along a scale, Pearsonian correlations were used in determining the degree of relationship between periodontal disease and the personality factors. Because of the

small number of intervals on both scales, correction for broad categories was applied.* Significance for these correlations was computed by the usual critical-ratio procedures. When the personality data were distributed into only two intervals, the periodontal data were also dichotomized to produce 2×2 tables, and tetrachoric correlations used to determine relationships. χ^2 was then computed for significance.†

RESULTS

Table 1 presents those objective, personal history, and personality factors which show correlations‡ of 0.30 or more with periodontal disease. These are all significant at the 0.05 level or better. A number of other rated variables showed statistically significant correlations with periodontal disease. All these correlations were in the 0.20's, and

TABLE 1

RELATIONSHIPS BETWEEN PERIODONTAL DISEASE AND PERSONALITY FACTORS

(Correlations above 0.30)

Var. No.	Variable	No. Cases	Correlation with Periodontal Disease	Significance*
1.....	Age	101	.50	$P<0.001$
5.....	Broken home	100	.51†	$P<0.01$
22.....	Marital adjustment	64	.39	$P<0.01$
32.....	Somatization	96	.33†	$P<0.05$
MMPI.....	Hysteria score	65	.32	$P<0.02$

* Significance refers to the likelihood that there is a true correlation between the variables in the population from which the sample is drawn. Thus, $P < 0.001$ means that there is less than one chance in a thousand that the two variables are not correlated in the population; $P < 0.05$ means less than five chances in 100, or one in 20; etc.

† Tetrachoric correlations (others are Pearsonian).

hence not high enough to do much more than indicate a trend. They are the following: rejection by family ($r = 0.27$); rigidity ($r = -0.27$); smoking ($r = 0.26$); tension $r = 0.25$); general adjustment ($r = 0.25$); friction with family ($r = 0.23$); lack of drive toward achievement ($r = 0.23$); lack of energy ($r = 0.23$); amount of hostility ($r = 0.22$); anxiety, guilt ($r = 0.21$); and repressiveness ($r = 0.20$).

The great majority of correlations, even those that did not achieve statistical significance, were positive. This may reflect a slight, but positive, relationship between general personality adjustment and periodontal disease, or it may reflect, for the personality factors, a halo effect in the ratings, or both. That the correlations in general were low may be due in part to the unreliability of the measures.

* Peters and Van Voorhis, *Statistical Procedures and Their Mathematical Bases* (New York: McGraw-Hill Book Co., Inc., 1940).

† All χ^2's were computed with Yates's correction.

‡ The coefficient of correlation ranges from -1 (perfect inverse relationship) through 0 (no relationship) to $+1$ (perfect positive relationship). The predictive value of the coefficient of correlation is approximately its square. This a coefficient of correlation of 0.50 predicts approximately 25 per cent of the total variance being described.

It will be noted that no significant correlation was found between diagnosis* and periodontal disease. This would argue against the likelihood that there is any close relationship between general adjustment and periodontal disease and also against the likelihood that the correlations were made spuriously high by lumping together the patients and non-patients into one group. Although an effort was made to conceal from the periodontist whether a given subject was a patient or a non-patient, this effort was not always successful. The lack of correlation between periodontal disease and psychiatric diagnosis offers assurance that the measures of periodontal disorder are not contaminated by the known psychiatric status of the subject.

TABLE 2

RELATIONSHIPS BETWEEN PERSONALITY FACTORS AND TWO COMPONENT MEASURES OF PERIODONTAL DISEASE

VAR. NO.	VARIABLE	CORRELATIONS*		
		A	B	C
1.....	Age	0.50	0.55	0.23
5.....	Broken home	0.51†	0.51†	0.50†
10a.....	Smoking	0.26	0.29	0.10
13.....	Rejection by family	0.27	0.27	0.18
15.....	Friction with family	0.23	0.39	0.13
22.....	Marital adjustment	0.39	0.43	0.16
24.....	Drive toward achievement	0.23	0.25	0.07
27.....	Anxiety, guilt	0.21	0.23	−0.13
28.....	Repressiveness	0.20	0.11	0.25
29.....	Rigidity	−0.27	−0.09	−0.24
30.....	Hostility (amount)	0.22	0.43	−0.03
32.....	Somatization	0.33†	0.41†	0.24†
33.....	Lack of energy	0.23	0.21	0.23
34.....	Tension	0.25	−0.01	0.04
35.....	General adjustment	0.25	0.24	0.09
MMPI.....	Hysteria score	0.32	0.09	0.10

* A = correlation with combined measure, from Table 1; B = correlation with average amount of bone recession per septum; C = correlation with average depth of pocket per septum.

† Tetrachoric correlations.

The criterion measures.—The question naturally arises whether or not some measure of periodontal disease other than the one arbitrarily selected might not yield closer relationships with personality factors. To gain information on this point, the two component parts of the "periodontal score" were correlated separately with each of the personality factors which showed significant relationships with the combined measure. The two components, as noted above, are (1) average amount of bone recession per septum as estimated from X-rays and (2) average depth of pocket per septum. The correlation between these two measures is 0.61 ($P < 0.001$).

Table 2 shows the results obtained. It will be noted that the estimates of bone recession yielded, on the average, much higher correlations with personality factors than did average depth of pocket alone, despite the fact that the former is a subjective measure and depth of pocket is objective. In fact, of the sixteen factors in the list, estimates of

* In investigating diagnosis, the subjects were divided into three groups—normal, neurotic, and psychotic; they were also divided into two groups—patient and non-patient.

bone recession yielded a correlation as high as, or higher than, the combined measure in ten. In only two instances was it higher for depth of pocket alone; and in only four was the combined measure superior to either of the two components.

It is clear that amount of bone recession, as here measured, is more closely related to personality than is depth of pockets. The fact that the combined measure yielded two correlations of significance that did not show up with either component and that depth of pocket yielded two which did not appear with bone recession alone may be a function of chance variability; but, on the other hand, these findings suggest that the pattern of personality factors manifesting a relationship with periodontal disease may depend on the aspect of periodontal pathology that is emphasized in the criterion measure used. In order to test this hypothesis and to find the measure of periodontal disease that will evidence the maximal relationship with personality, further investigation is needed.

DISCUSSION

It is not surprising to find age yielding a substantial correlation with periodontal disease; it has long been known that periodontal disorders are related to age. Other correlations, such as those with broken home and marital adjustment, disclose less obvious relationships. Why should the fact that a person did not live with his own parents continuously before he was sixteen be reflected in his oral pathology twenty years later? Or why should a person who has experienced much discord in his marriage be more prone than others to periodontal disease? The correlation between somatization and periodontal disease suggests that the people who are most likely to show periodontal disorders are, at least in part, the same people as those who tend to develop physical complaints and symptoms as a result of their personality conflicts and who hence tend to develop psychosomatic illnesses.

The independent findings from the Minnesota Multiphase Personality Inventory (MMPI) support this interpretation. The only diagnostic scale on the instrument to show a significant correlation with periodontal disease was hysteria. The next highest MMPI correlation, and the only other one approaching significance, was that with hypochondriasis. These two scores are characteristically elevated in the MMPI records of psychosomatic patients and are often the highest scores for such patients.

The data collected in this study do not suffice to establish definitely that there is an important relationship between general personality adjustment and periodontal disease. Any such relationship that may exist is probably not a close one. The findings do suggest, however, that periodontal disease tends to be associated more closely with broken home and marital adjustment. The findings also indicate the likelihood that there is a psychogenic component or psychosomatic process involved in at least some periodontal disease.

SUMMARY

Sixty-two psychiatric patients and 40 non-patients were given a thorough oral examination to evaluate the amount of periodontal disease present. They were also given a diagnostic personality inventory (MMPI) and were rated on thirty-five objective, personal history, and personality variables. The data were explored to investigate the relationships between periodontal disease and personality factors.

The results indicate a positive relationship between periodontal pathology and age, marital adjustment, broken home, somatization, the MMPI hysteria scale, and a number of other variables.

It is concluded that certain personality factors, or types of adjustment, tend to be associated with periodontal disease and that further exploration of these relationships is desirable.

The authors are indebted for aid and encouragement to Dr. Willard Fleming dean, School of Dentistry, University of California; Dr. Karl Bowman, professor of psychiatry, University of California, medical professor for the state of California, Department of Mental Hygiene; Dr. James Hopper, associate professor of medicine, University of California; and Dr. Harold Ray, professor of periodontology and chairman of the division, University of California School of Dentistry.

REFERENCES

1. CAMPBELL, D. G. Psychosomatic Mechanisms in Oral Diseases, *Am. J. Orthodont. & Oral Surg., Sec. Oral Surg.*, **31**:440, 1945.
2. RYAN, E. J. *Psychobiologic Foundations in Dentistry*, p. 49. Springfield: Charles C Thomas, 1946.
3. BURSTONE, M. S. The Psychosomatic Aspects of Dental Problems, *J.A.D.A.*, **33**:862, 1946.
4. LANDA, J. S. Some Psychosomatic Aspects of Medicine, *D. Items of Interest*, **69**:99, 1947.
5. MILLER, S. C. *Textbook of Periodontia*, p. 87. Philadelphia: Blakiston Co., 1947.
6. JONES, H. S. Emotional Stress and Gingivitis, *Oral Hygiene*, **41**:657, 1951.
7. ROSENBERG, M. Psychosomatic Factors in Periodontal Disease, *New York Univ. J.D.*, **9**:273, 1950.
8. ROTH, H. and STONE, A. Psychosomatic and Nutritional Factors Related to Recurrent Necrotizing Ulcerative Gingivitis, *J.A.D.A.*, **42**:474, 1951.
9. BELL, W. E. The Psychosomatic Concept of Oral Disease, *Baylor D. J.*, **3**:4, 1953.
10. GLICKMAN, I. *Clinical Periodontology*, p. 418. Philadelphia and London: W. B. Saunders Co., 1958.
11. MOULTON, R. Psychologic Problems Associated with Complete Dental Service, *J.A.D.A.*, **33**:476, 1946.
12. THOMPSON, C. E. Suggested Approach for the Study of Psychosomatic Dentistry, *D. Items of Interest*, **75**:486, 1953.
13. WALSH, J. P. Psychogenic Symptoms in Dental Practice, *Oral Surg., Oral Med. & Oral Path.*, **6**:437, 1953.
14. ZEIFERT, M. Psychiatric Aspects of Dentistry, *J. Calif. State Assoc. & Nevada D. Soc.*, **29**:316, 1953.
15. BALTERS, W. Psychology and Dentistry, *Internat. D. J.*, **4**:224, 1953.
16. RAGINSKY, B. B. Psychosomatic Dentistry, *J. Canad. Dent. A.*, **20**:479, 1954.
17. ZAIDENS, S. H. Psychosomatic Disorders of the Mouth, *New York. J. Den.*, **24**:218, 1954.
18. WEISS, J. K. Some Psychological Aspects of Dentistry, *Tufts D. Outlook*, **24**:2, 1951.
19. MANHOLD, J. H., and MANHOLD, V. W. Preliminary Report on the Relationship of Psychosomatics to Oral Conditions. II. Relationship of Personality to Periodontal Conditions, *J. D. Res.*, **30**:459, 1951.
20. MANHOLD, J. H. Report of a Study on the Relationship of Personality Variables to Periodontal Conditions, *J. Periodont.*, **24**:248, 1953.
21. MANHOLD, J. H., and ROSENBERG, N. Study of the Possible Relationship of Personality Variables to Dental Cavities, *J. D. Res.*, **33**:357, 1954.
22. MANHOLD, J. H., and JONES, M. B. Preliminary Study of Social Attitudes in Relation to Dental Caries Formation, *Washington Univ. D. J.*, **22**:41, 1956.
23. GOLDBERG, H., AMBINDER, W. J., COOPER L., and ABRAMS, A. L. Emotional Status of Patients with Acute Gingivitis, *New York D. J.*, **22**:308, 1956.
24. MOULTON, R., EWEN S., and THIEMAN, W. Emotional Factors in Periodontal Disease, *Oral Surg., Oral Med. & Oral Path.*, **5**:833, 1952.
25. RAY, H. Periodontal Index, personal communication.

Cutting Studies at Air-Turbine Speeds

*J. O. SEMMELMAN, P. R. KULP, and L. KURLANSIK**

Dentists' Supply Company of New York, York, Pennsylvania

In November, 1957, the Dentists' Supply Company undertook mass production of the first air-turbine dental contra-angle handpiece. This development made possible far more rapid and painless cavity preparation and also opened the way to new research approaches to the subject of cutting hard tissue.

Hartley[1] had previously investigated cutting rates with belt-driven rotary instruments up to 15,000 rpm, and Peyton[2] had extended the coverage of speeds up to 200,000 rpm under fixed loads. No one had measured the actual speed of air-turbine handpieces during the cutting operation or the conditions of torque and load necessary for optimum cutting at the new, higher speeds.

The present work, conducted during 1958, had as its purpose an understanding of the relative functions of speed and of torque in the cutting operation. The study was limited in scope to air-turbine contra-angles, where power and speed variations could be covered thoroughly and easily by control of the air pressure and transverse loading.

Measurement of rotational speed during actual cutting was required for each condition of power and load. This required that the handpiece be operated in a fixed position so that an optical tachometer could be kept focused on the rotating shaft while the specimen being cut was moved against the rotating bur under known loads.

The equipment assembly (Figs. 1 and 2) centered around the fixed mounting for the handpiece. The shafts of the cutting instruments were blackened on one side and polished on the other, alternately to interrupt and reflect the focused light beam. The electronic tachometer† provided the light source and photocell to pick up the reflections. The frequency of photocell impulses was counted by a meter.‡ An oscilloscope§ was used to indicate maximum signal strength, as this was necessary for accurate counting.

The system of moving the specimen under load included an adjustable stand with grips to hold the sample being cut. This was mounted on a nearly frictionless carriage which was pulled by a dead-weight loading system connected through a series of pulleys.

A uniform specimen thickness was necessary to maintain constant cutting area and load per unit area; all specimens therefore were prepared in $\frac{1}{16}$-inch thickness (Fig. 3). Preliminary studies of metal specimens established that the length should be 1 inch $\pm \frac{1}{4}$

Received for publication May 10, 1960.

* The authors are members of the Research Department of the Dentists' Supply Company, York, Pennsylvania. However, Mr. Kurlansik is no longer associated in this capacity.

† Berkeley, Model 331, Berkeley Division, Hickok Instruments, Inc., Richmond, California.

‡ Berkeley Eput, Model 7150 B., Berkeley Division, Hickok Instruments, Inc., Richmond, California.

§ Hickok Model 770, The Hickok Electrical Instrument Co., Cleveland, Ohio.

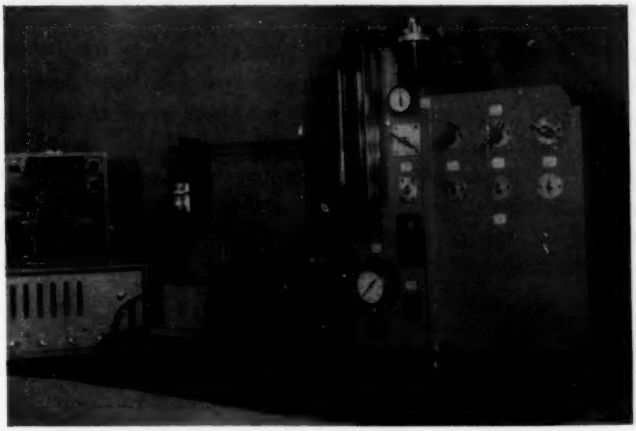


FIG. 1.—The equipment assembly centered around the fixed mounting for the handpiece

FIG. 2.—The mounting for the handpiece

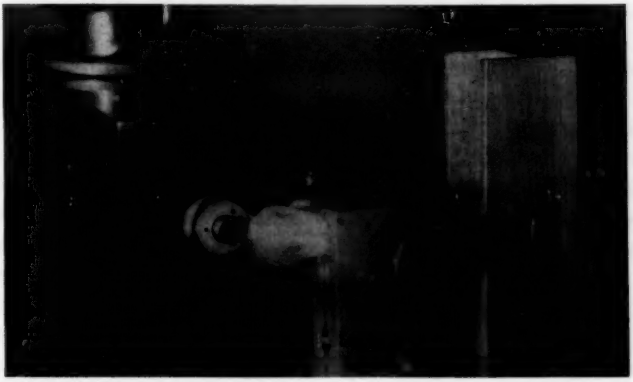


FIG. 3.—Detail of Fig. 2, showing specimen prepared in 1/16-inch thickness

to provide adequate stock for cutting, to avoid vibrational effects or "chatter," and to correlate with tooth sizes. Natural tooth specimens were $\frac{1}{16}$-inch mesiodistal sections of freshly extracted upper incisors. It might be noted that the orientation of the section relative to the tooth structure did not have a significant effect on the cutting data; this was surprising at first but was eventually attributed to the small size of individual chips relative to the tubular tooth structure.

Test technique in preliminary studies of brass cutting included determining the original dry weight of the specimen; cutting for 15 seconds, during which the bur rotational

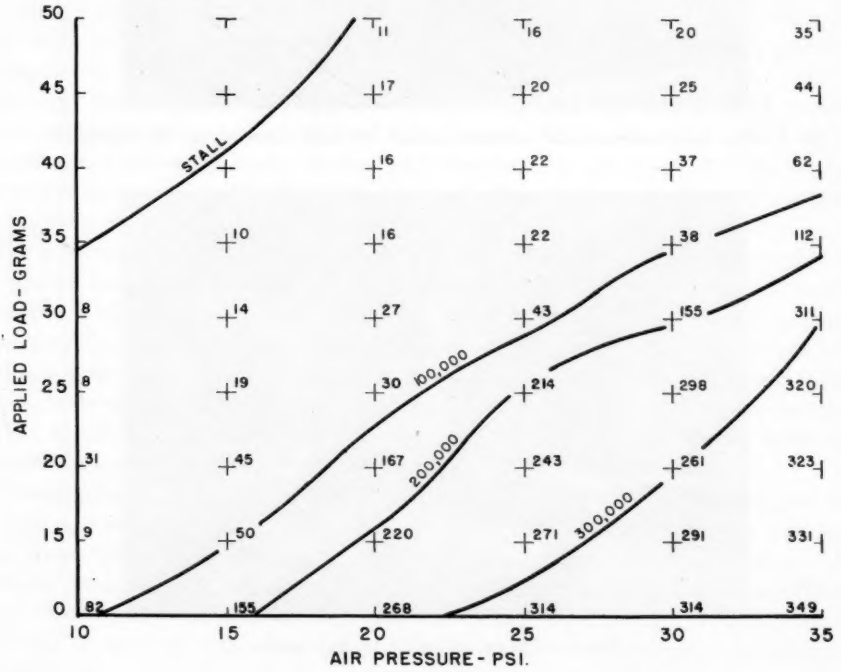


FIG. 4.—No. 557 bur, cutting brass dry at 1,000 RPM

speed was recorded; reweighing the specimen; and calculating the material removal rate. In subsequent studies of natural teeth, sections were kept in water until immediately before use, when they were blotted with a paper towel, dried with an air blast for 15 seconds, and weighed. Water cooling was used during the cutting of teeth, the duration of this operation varying from 6 to 15 seconds, depending on the size of tooth and the type of instrument used. Accuracy of weighing and reweighing in all cases was determined to be $\pm$ 1 mg., and all material-removal rates were calculated and reported in milligrams per minute.

In the basic study of brass cutting, the type of brass used was identified as "free cutting quality," and the cutting tool was a No. 557 carbide bur. All data reported represent an average of five separate tests. Actual rotational speeds are summarized in Figure 4. Various air pressures supplied to drive the turbine are plotted along the ab-

scissa, the applied transverse loads on the ordinate. The average rotational speed resulting from each combination of rotational and transverse pressure is reported in thousands of revolutions per minute, very much as elevations would be above a two-dimensional plane. Isobars connect points of equal rotational speed and are most easily visualized as similar to topographic contours. Under zero-load conditions, free-running speeds are seen to increase with increasing air pressure throughout the range studied. Data values were inconsistent below 15 gm. applied transverse load, as differences between static and dynamic friction in the loading system caused vibratory contact or

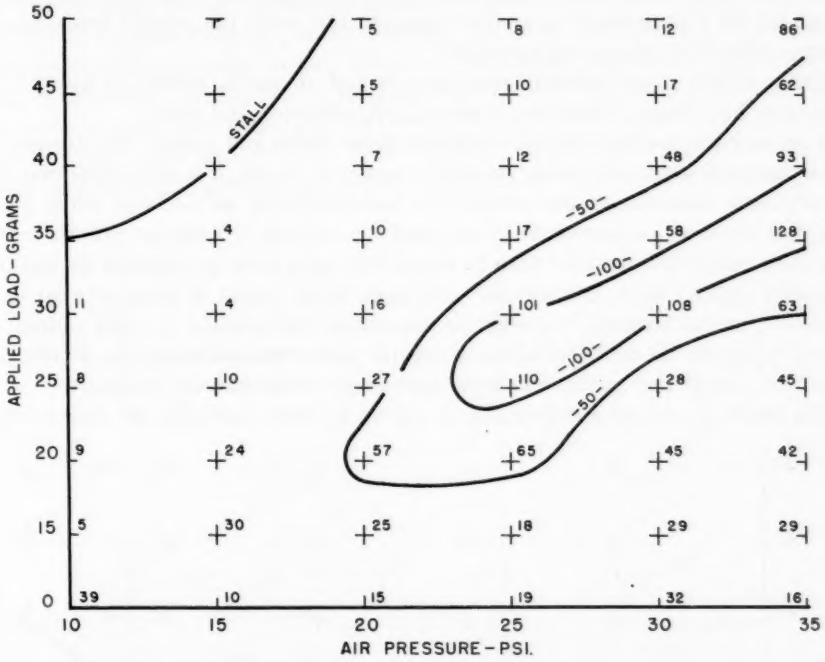


FIG. 5.—No. 557 bur, cutting brass dry. Material removal in mg/min

"chatter" of the specimen. Through the rest of the field, however, increasing load caused decreasing speed, and there is a general pattern of equal-speed isobars from light transverse loads with low air pressures to heavier applied loads and greater air pressures. The air turbine, of course, stalls completely with excessive application of load.

Simultaneously measured material-removal rates are reported for the same test conditions in Figure 5. In this case, the cutting rate is reported in milligrams per minute for each combination of rotational pressure and transverse load. It is apparent that cutting rates follow an inclined pattern similar to rotational speed and that optimum cutting is secured from 25-psi air pressure and 25-gm. applied load to 35-psi air pressure and 35-gm. applied load. Comparing Figures 4 and 5, the optimum rate of brass cutting is seen to result from rotational speeds between 150,000 and 220,000 rpm.

A brief discussion of the functions of applied loading relative to an air turbine may assist in further explanation of the data. Transverse load or applied pressure has two

effects—(1) causing an indentation or bite of the bur flutes and (2) controlling the rotational speed. Thus near-stalling loads result in large bites being taken but with insufficient frequency for maximum cutting effectiveness. Conversely, near-zero loads permit the highest rotational speed or frequency of flute impacts, but the indentation of each flute is too small to produce a high cutting rate. The optimum compromise between these extremes is achieved at an intermediate rotational speed, which, for any given air-supply pressure, must be maintained by very delicate control of applied loads. The sensitivity of the air turbine to optimum loading is seen to be approximately 10 gm. under test conditions. Comparison with manual cutting rates indicates that human sensitivity or control must be at least equal to this, with the manual load-adjusting process being continuous and automatic.

The optimum speed and load requirements are, of course, influenced by such tool factors as flute design, sharpness, diamond grit, diameter, and others.

A relationship having been established between speed and cutting effectiveness, the same data were next re-examined relative to power or torque. It is interesting that there is very little difference in the cutting rate between 25-psi air pressure under 25-gm. load and 35-psi air pressure with 35-gm. load. Less energy is being put into the cutting operation in the first instance than in the second, both from pressurized air and from manually applied force, but virtually the same work output is being secured in the form of material removal. To assist in visualizing this relation of work output and energy input for all test conditions, a relative index was calculated by dividing the material-removal rate by the two input pressures—rotational and transverse.

The result is portrayed graphically in Figure 6, which indicates the desirability of

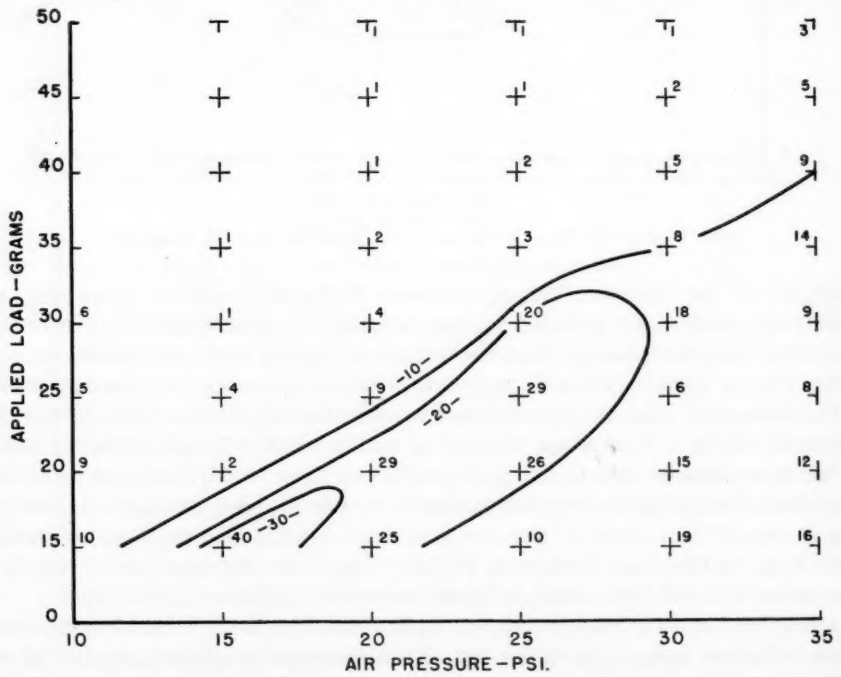


FIG. 6.—No. 557 bur, cutting brass dry. Index of material removed divided by air pressure and load

minimum pressure, although, of course, a reasonably rapid cutting rate must also be maintained. The ideal compromise between efficiency and effectiveness for an air turbine is along the 200,000 rpm isobar at 25-psi operating air pressure, which requires the minimum use of pressure to accomplish a high removal rate. Lower air pressures and lighter loads may be preferable in finishing operations, where lower removal rates are desired, where it may be necessary to work without a coolant and efficiency and heat generation are more important, and where smaller bites per bur flute may contribute to better surfaces.

A further view of the relationship between torque and cutting results by referencing both back to speed (Fig. 4). Air turbines are known to have inherent high speed and low torque characteristics; in fact, speed and torque bear a generally inverse relationship to each other. Within the air-turbine range studied here, increasing the air-supply pressure increased the speed and the *potential* torque, but no actual torque was delivered or any work accomplished until a load was applied. Full realization of torque from air pressure is achieved only under stalling loads where there is no cutting action.

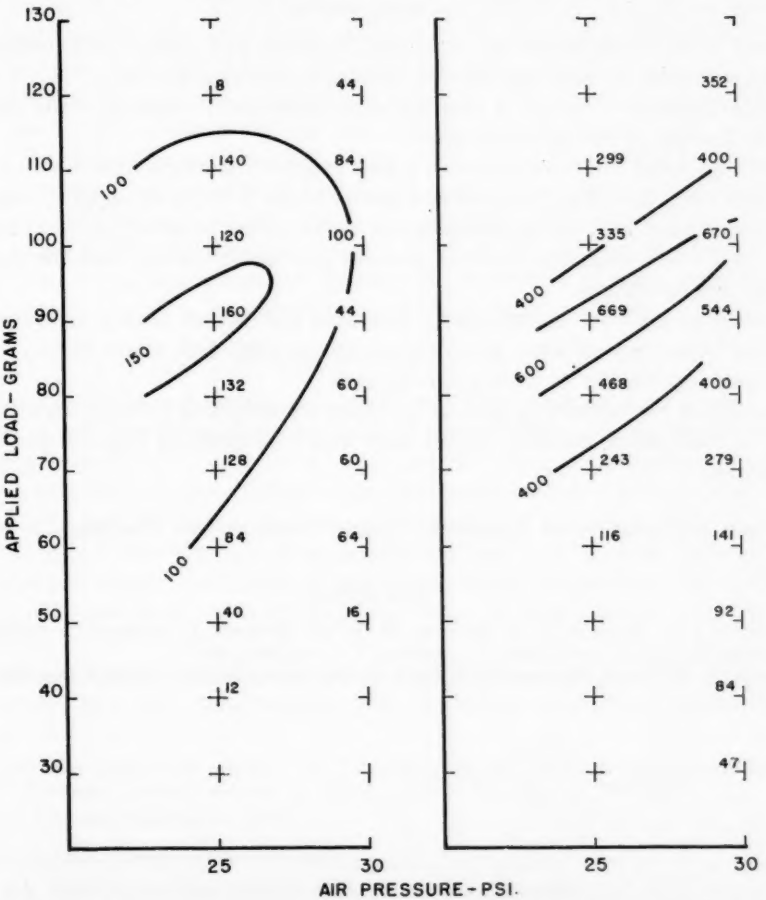


FIG. 7.—Natural tooth removal (mg/min). *Left,* No. 107 diamond, wet; *right,* No. 557 bur, wet

While increasing the air pressure serves to increase the stalling potential or apparent torque, the practical effect is to require the operator to bear down harder to achieve the optimum rotational speed and torque delivery. The use of one force to counteract another above the speed-torque level required for effective cutting seemed to result in some loss of tactile sensitivity and logically would also contribute to heat generation.

With this view of the mechanics of air-turbine cutting in brass, a similar study was performed for verification on freshly extracted natural teeth. As many as ten tooth specimens were measured to arrive at average data for reporting. Test coverage was increased to include diamond instruments as well as burs and cutting with a coolant, and pressure and load variables were restricted accordingly. Inclined patterns of material removal rates were observed (Fig. 7) smiliar to those in cutting brass. However, higher applied loads were required to achieve optimum cutting of tooth substance (80–100 gm. at 25–30 psi). Speeds were not graphed, but optimum cutting was measured as occurring between the 200,000- and 250,000-rpm isobars. It should be noted also that tooth material-removal rates were considerably higher than with brass.

CONCLUSIONS

Speed is a very important factor in dental cutting, and optimum air-turbine speed can be controlled by manually applied load with surprising accuracy.

Actual delivery of torque is also manually controlled by applied loads and is an inverse function of the operating speed.

Optimum speed can be secured over a wide range of conditions from low air pressure and light loads to high air pressure and heavier loads. A 25-psi air-supply pressure and a 90-gm. applied load are recommended as the minimum pressure condition for rapid removal of tooth structure. Lower air pressure and lighter loading would be preferable for finishing operations.

Stalling or apparent torque can be increased with higher air-supply pressure but requires higher applied loads for optimum cutting rates and, above 25 psi, does not contribute significantly to cutting effectiveness.

The harder the material being cut, the higher the indenting pressure required; however, the more brittle materials benefit more from high speed, as they cut more cleanly and rapidly.

The authors acknowledge the assistance of Richard Plowman in recent rechecking of tooth-cutting data.

REFERENCES

1. Hartley, J. L., Hudson, D. C., Sweeney, W. T., and Dickson, G. Methods for Evaluation of Rotating Diamond-abrasive Dental Instruments, *J.A.D.A.*, **54**:5, 1957.
2. Peyton, F. A. Current Developments in Tooth Cutting Instruments, *New York J. Den.*, **28**:5, 1958.

Thermal Conductivity of Tooth Structure, Dental Cements, and Amalgam

R. G. CRAIG and F. A. PEYTON

School of Dentistry, University of Michigan, Ann Arbor, Michigan

The thermal conductivity of human dentin has been reported by Lisanti and Zander,[1] Simeral,[2] Phillips, Reinking, and Phillips,[3] and Soyenkoff and Okun[4] to be 2.29×10^{-3}, 2.35×10^{-3}, 0.257×10^{-3}, and $0.96–1.07 \times 10^{-3}$ cal/sec/cm^2/°C/cm, respectively. The thermal conductivity of human enamel has been reported by Soyenkoff and Okun[3] to be 1.55×10^{-3}.

More limited data are available for zinc phosphate and silicate cements. Simeral[2] reported values of 2.81×10^{-3} for zinc phosphate and 2.00×10^{-3} for silicate cements. Phillips and co-workers[3, 5] at different times listed the thermal conductivity of zinc phosphate cement to be $3.91–5.37 \times 10^{-3}$ and $0.311–0.388 \times 10^{-3}$ cal/sec/cm^2/°C/cm, which represents the centimeter, gram, second (c.g.s.) units of measurement. Silicate cement was found to have a thermal conductivity of 0.458×10^{-3} c.g.s. units.[5] A survey of the dental literature revealed that no values have been reported for amalgam.

All the literature values indicated that dentin, enamel, zinc phosphate cements, and silicate cements were good thermal insulators, although the numerical values for the various materials often disagreed by a factor of 10. It was the purpose of this investigation to establish more accurately the thermal conductivity of tooth structure and dental cements, as well as to determine a value for dental amalgam.

MATERIALS AND METHODS

Specimen preparation.—Dentin and enamel specimens were cut from human teeth by means of a diamond-core drill mounted on a jeweler's lathe. The cylindrical blanks obtained were held in the lathe by a chuck, and the sides of the cylinder were cut down by a tungsten carbide tool. The dentin specimens were cut to a diameter of 0.234 inch (15/64″) and the enamel specimens to a diameter of 0.156 inch (5/32″). These finished cylinders were then placed in appropriate collets in the lathe and the ends ground flat and parallel by a grinding attachment and an India stone. The thickness of the enamel specimens was 0.030 inch, and those for dentin varied from 0.045 to 0.060 inch.

The zinc phosphate* and silicate† cement specimens were prepared by placing the mixed cement in a split stainless-steel mold, which was 0.234 inch in diameter and

This study was supported in part by U.S.P.H.S. research grant D-462 from the National Institute of Dental Research, National Institutes of Health, and the University of Michigan.

Received for publication May 12, 1960.

* The L. D. Caulk Company, Tenacin Cement.

† The S. S. White Dental Manufacturing Co., Improved Filling Porcelain.

0.12 inch thick. The zinc phosphate inlay seating consistency mix contained 1.1 gm. of powder for 0.5 cc. of liquid, and thick cement base mix contained 1.85 gm. of powder for 0.5 cc. of cement liquid. The consistency of the silicate cement samples was 1.43 gm. of powder for 0.4 cc. of cement liquid. After preparation, the samples were stored in distilled water until the thermal conductivity was determined, which varied from 1 to 7 days after preparation.

The amalgam specimens were condensed in stainless-steel molds and had dimensions of 0.234 inch in diameter and a thickness of either 0.06 or 0.12 inch. The manufacturer's* recommended proportions were used, and normal (8–10-lb.), heavy (10–12-lb.), or light (4–8-lb.) condensation pressure on a 2-mm. condenser was used. The surfaces of most of the specimens were finished by lightly polishing with 600 A silicon carbide paper. A few of the specimens were used with the surface finish obtained by trimming only with a razor blade.

Thermal conductivity equipment.—The thermal conductivity was determined with equipment which was essentially a modification of that reported by Lisanti and Zander.[1] The specimens were placed between two pure copper rods and were held firmly in position by the action of a spring. The copper rods used were the same diameter as the specimens, and each was 6.5 cm. long. One end of the rod was ground flat with the jeweler's lathe, and the other end was threaded so that it could be screwed into the heating and cooling unit. Minute holes were drilled in the rods 1 mm. from the sample end and at intervals of 1 cm. Thus each rod had seven holes spaced at 1-cm. intervals into which were inserted copper-constantan thermocouples made from 28-gauge wire. The threaded end of one rod was screwed into a copper block, which could be electrically heated by current from a 6-volt storage battery. The other rod was screwed into another copper block, which was cooled by circulating water. The cooling water was taken from a thermostatically controlled storage tank which maintained a constant pressure.

Thermal conductivity measurement.—The rod assembly, including supports, was contained in a plywood box, having a 16-inch edge length. The lower portion of the box was packed with glass-wool insulation, while sections of polystyrene foam were used in the upper parts of the box. The foam sections could be easily removed to allow for changing thermal conductivity samples.

The thermal conductivity sample was placed between the copper rods, using a film of glycerine on each surface to insure adequate contact. The entire system was allowed to reach a steady-state condition, where the heat flow in equaled the heat flow out. Under these conditions, the temperature of each position remained constant with time. Then the temperature at the various positions along the copper rods was measured by the thermocouples and a potentiometer.† The potentiometer had a sensitive scale from 0 to 16 millivolts, graduated in 0.01 millivolt. The temperature could be measured to $\pm 0.05^\circ$ C. with this equipment. An extrapolation of the plot of the temperature against distance along the copper rods permitted the determination of the surface temperature on the hot and cold sides of the conductivity specimen.

In order to establish whether or not steady-state conditions had been attained, which took from 16 to 20 hours, the temperature of the thermocouple positions was determined at 1–2-hour intervals. When two successive runs gave the same temperature

* The S. S. White Dental Manufacturing Co. † Leeds and Northrup Co.

values, it was assumed that the system had reached a steady state. After steady state had been established, a minimum of four separate determinations, at 1-hour intervals, was made of the thermal conductivity of each of the specimens.

Thermal conductivity calculation.—Under steady-state conditions the quantity of heat flowing through the copper rod, q_1, is represented by equation, (1),

$$q_1 = k_1 A_1 \left(\frac{\Delta t_1}{X_1}\right), \tag{1}$$

where k_1 is the thermal conductivity of copper, A_1 is the area of the copper rods, Δt_1 is the change in temperature along the copper rod, and X_1 is the distance over which this temperature change occurs. The quantity of heat flowing through the specimen may be represented by $q_2 = U\Delta t_{2,3}$, where U is equal to the following expression:

$$U = \frac{1}{(X_2/k_2A_2) + (X_3/k_3A_3)}. \tag{2}$$

The terms X_2 and X_3 are the sample and glycerine thicknesses, respectively, while k_2 and k_3 are the thermal conductivities of the sample and glycerine, and A_2 and A_3 are the areas of the sample and glycerine film, respectively. The term $\Delta t_{2,3}$ represents the temperature drop across the sample and the glycerine films.

The qualities q_1 and q_2 may be equated under conditions of steady state, as indicated in equation (3),

$$k_1 A_1 \left(\frac{\Delta t_1}{X_1}\right) = \frac{\Delta t_{2,3}}{(X_2/k_2A_2) + (X_3/k_3A_3)}. \tag{3}$$

All the quantities in equation (3) are known, except k_2, the thermal conductivity of the specimen, and thus it may be calculated. Equation (3) has been simplified for ease of calculating the thermal conductivities of unknown materials, as shown in equation (4):

$$k_2 = \frac{k_1 k_3 d_1^2 (X_2 \text{ slope}_1)}{k_3 d_2^2 (\Delta t_{2,3}) - k_1 d_3^2 X_3 (\text{slope}_1)}. \tag{4}$$

For the sake of simplicity, the squares of the diameters of the rods, specimen, and glycerine film were used, rather than the areas. Likewise, the slope of the temperature versus distance plot along the copper rod on the cold side was substituted for t_1/X_1. If the average of the slope on the hot and cold slides were used, this would increase the thermal conductivity of dentin from the reported value of 1.4×10^{-3} to 1.7×10^{-3} cal/sec/cm^2/°C/cm. For each thermal conductivity measurement, the terms in parentheses were required. The sample thickness, the slope of the temperature versus distance plot, and the temperature drop across the specimen, therefore, were needed in order to calculate the thermal conductivity.

The right-hand term in the denominator of equation (4) is the correction for the glycerine film on each side of the specimen. Values for X_3, the double film thickness, were determined by using a copper specimen and were checked by using a glass* specimen approximately the thickness of the unknown specimen. The glycerine film

* Corning Glass Company, Pyrex glass.

thickness, using the 0.234-inch diameter copper rods, was 20 μ for all samples from 0.045 to 0.12 inch thick. The 0.156-inch diameter copper rods were used only for the smaller enamel specimens, and a film thickness of 50 μ was obtained. This increase in film thickness presumably was a result of using copper rods 60 mm. in length rather than the usual length of 65 mm. A significantly lower spring pressure was exerted on the samples when the shorter copper rods were used, and therefore a thicker glycerine film was observed.

Glass* specimens of various thickness, with a thermal conductivity of 2.7×10^{-3} cal/sec/cm^2/°C/cm were used as standards throughout this study, and ten standardization runs were made. A maximum error of 3 per cent was observed in the standardization runs. Seven of the ten standardization runs were within 1.5 per cent of the theoretical value ($\pm 0.03 \times 10^{-3}$ c.g.s. units). It was observed that slightly better

TABLE 1

THERMAL CONDUCTIVITY OF DENTIN AND ENAMEL*

SAMPLE	DENTIN		ENAMEL
	Parallel to Tubules ($K \times 10^3$)	Perpendicular to Tubules ($K \times 10^3$)	Parallel to Rods ($K \times 10^3$)
1	1.43±0.03	1.45±0.04	2.19±0.04
2	1.53±0.02	1.65±0.04	2.20±0.06
3	1.40±0.04	1.12±0.02	2.16±0.06
4	1.31±0.03	1.25±0.03	2.10±0.07
5	1.40±0.04	1.54±0.05	2.55±0.04
6	1.32±0.05	1.38±0.03	2.10±0.03
7	1.25±0.03	1.34±0.04	2.29±0.09
8	1.30±0.04	.....	.....
Mean..	1.36±0.07×10^{-3}	1.39±0.12×10^{-3}	2.23±0.11×10^{-3}

* Determined at an average temperature of 50° C.; reported in cal/sec/cm^2/°C/cm.

thermal conductivity values for glass were obtained if the slope of the temperature versus distance plot on the cold side of the specimen was used. The slope on the hot side was always slightly higher and, if averaged with the slope on the cold side, gave somewhat higher thermal conductivity values. The higher rate of change of temperature on the hot side of the sample was presumably due to greater heat loss by radiation, since the rod temperature was approximately 45° C. higher than the ambient temperature of the insulation. Radiation effects may be neglected in the case of the copper rod on the cold side, since the temperature was within a few degrees of the ambient temperature.

RESULTS

The thermal conductivity values for human dentin and enamel are listed in Table 1. Dentin specimens having the tubule direction parallel to the heat flow resulted in an average thermal conductivity for eight samples of 1.36×10^{-3} cal/sec/cm^2/°C/cm and an average deviation from the mean of $\pm 0.07 \times 10^{-3}$. Dentin samples with the tubule direction generally perpendicular to the heat flow had an average value of

* Corning Glass Company, Pyrex glass.

$1.39 \pm 0.12 \times 10^{-3}$ c.g.s. units. These data show that tubule direction does not influence the thermal conductivity of dentin.

Human enamel had a higher thermal conductivity than dentin, with values ranging from 2.10 to 2.55×10^{-3} c.g.s. units. The mean value for enamel with the rod direction parallel to the heat flow was $2.23 \pm 0.11 \times 10^{3}$ c.g.s. units. It should be noted that the average deviation from the mean for a single sample of dentin or enamel was less than the average deviation calculated by averaging the thermal conductivities of all samples of a similar type. This suggests an actual variation in the thermal conductivity from one tooth to another or possibly variations in sample preparation.

The zinc phosphate cement specimens, prepared from an inlay or crown-and-bridge consistency mix and measured in an essentially wet condition, resulted in a mean value of 2.5×10^{-3} cal/sec/cm^2/°C/cm and an average deviation from the mean of $\pm 0.3 \times 10^{-3}$ c.g.s. units, as shown in Table 2. The samples prepared from the base-

TABLE 2

THERMAL CONDUCTIVITY OF ZINC PHOSPHATE AND SILICATE CEMENTS

Sample	Zinc Phosphate Cement*		Silicate Cement†		
	Inlay Mix‡ ($K \times 10^3$)	Base Mix§ ($K \times 10^3$)	Days after Mix	1st Day of Run ($K \times 10^3$)	2d Day of Run ($K \times 10^3$)
1	1.93±0.09	3.72±0.10	1	1.90±0.03	1.82±0.04
2	3.10±0.10	3.46±0.01	2	1.76±0.04	1.57±0.06
3	2.65±0.10	2.91±0.02	3	1.57±0.02	1.73±0.03
4	2.54±0.06	2.89±0.03	5	1.70±0.05	1.75±0.03
5	2.53±0.09	2.80±0.09	5	2.02±0.01	1.81±0.07
6	2.14±0.07	2.56±0.09	7	2.20±0.06	2.03±0.02
Mean	$2.5 \pm 0.3 \times 10^{-3}$	$3.1 \pm 0.3 \times 10^{-3}$	.....	$1.9 \pm 0.2 \times 10^{-3}$	$1.8 \pm 0.1 \times 10^{-3}$

* Determined at an average temperature of 55° C., reported in cal/sec/cm²/° C/cm.
† Determined at an average temperature of 45° C., reported in cal/sec/cm²/° C/cm.
‡ 1.1 gm. powder to 0.5 cc. cement liquid.
§ 1.85 gm. powder to 0.5 cc. cement liquid.

consistency mixes had an average value of 3.1×10^{-3} c.g.s. units. Since the average deviation from the mean was again $\pm 0.3 \times 10^{-3}$ c.g.s. units, the data indicate only a slight difference between the thermal conductivity of inlay and base consistency mixes of zinc phosphate cements. Again, less variation was found between different runs from a single sample than between different samples.

The thermal conductivities of the silicate cement specimens also are listed in Table 2. Two values are given for each sample, the first representing the thermal conductivity during the first day in the thermal conductivity apparatus and the second value giving the conductivity during the second day. The average values of $1.9 \pm 0.2 \times 10^{-3}$ cal/sec/cm^2/°C/cm for the first day and $1.8 \pm 0.1 \times 10^{-3}$ c.g.s. units for the second day illustrate that little, if any, dehydration took place under the experimental conditions. The thermal conductivity of the silicate cement specimens was determined 1,2,3,5, and 7 days after preparation, and no trend was observed, possibly because of the variations obtained for different samples.

Dental amalgam was found to have an average thermal conductivity of 5.4×10^{-2} cal/sec/cm^2/°C/cm, as reported in Table 3. Considerable variation was observed from

sample to sample, as indicated by figures ranging from 4.43 to 6.87 $\times$ 10^{-2} c.g.s. units. Unfortunately, no trend in the figures could be attributed to condensation pressure, sample finish, amalgam alloy, or mercury content of the amalgam samples, since variations between samples were too large. Finished samples prepared by using normal condensation pressure had thermal conductivities from 4.43 to 6.87 $\times$ 10^{-2} cal/sec/cm^2/°C/cm; those prepared with heavy condensation pressure varied from 5.05 to 6.40 $\times$ 10^{-2}; and those condensed with light pressure ranged from 6.10 to 6.23 $\times$ 10^{-2} c.g.s. units.

It might be expected that samples having the highest mercury content would have the lowest thermal conductivity, but sample 13, a lightly condensed specimen with

TABLE 3

THERMAL CONDUCTIVITY OF DENTAL AMALGAM*

Sample†	Condensation Pressure	Sample Surface Finish	Mercury Content (Per Cent)	$K \times 10^2$
1	Normal	Unfinished	45.2	4.46±0.06
2	Normal	Unfinished	.....	4.64±0.05
3	Normal	Unfinished	47.0	5.52±0.06
4	Normal	Finished	46.4	5.40±0.06
5	Normal	Finished	.....	4.43±0.12
6	Normal	Finished	.....	4.47±0.13
7	Normal	Finished	.....	4.54±0.08
8‡	Normal	Finished	43.6	6.87±0.14
9‡	Normal	Finished	42.8	5.35±0.15
10	Heavy	Finished	43.3	6.40±0.14
11	Heavy	Finished	.....	6.33±0.06
12	Heavy	Finished	.....	5.05±0.07
13	Light	Finished	47.8	6.10±0.05
14	Light	Finished	.....	6.23±0.10
Mean	.....	.....	.....	5.4±0.7×10^{-2}

* Determined at an average temperature of 36° C., in cal/sec/cm^2/° C/cm.
† All samples except 8 and 9 were prepared from S. S. White Sigrens.
‡ Prepared from S. S. White New True Dental Alloy, fine cut.

the highest mercury content of 47.8 per cent, had a relatively high conductivity of 6.10 $\times$ 10^{-2} c.g.s. units. Also sample 9, which had the lowest mercury content of 42.8 per cent, resulted in a lower conductivity value of only 5.35 $\times$ 10^{-2} c.g.s. units. Table 3 includes other examples of the lack of correlation of manipulation of amalgam with thermal conductivity.

DISCUSSION

A summary of the thermal conductivity for dentin, enamel, zinc phosphate cement, silicate cement, and amalgam reported by various investigators is presented in Table 4. The values are listed chronologically for each material.

The figures listed for human dentin can be divided into three groups. The average thermal conductivity of 2.29 $\times$ 10^{-3} and 2.35 $\times$ 10^{-3} cal/sec/cm^2/°C/cm reported by Lisanti and Zander[1] and Simeral,[2] respectively, are in close agreement. These results are nearly twice the values of 1.35–1.39 $\times$ 10^{-3} found in this research with essentially the same procedures. The principal difference in their procedure was the use of the average of the slopes of the temperature versus distance plot for the hot and cold

copper rod in the thermal conductivity calculation, which would result in higher values. Improvement in sample preparation in the present study may also account for the difference in results.

The figure of 0.257×10^{-3} reported by Phillips and co-workers[5] is only one-fifth the value found in this study. This value, as well as their figures for zinc phosphate and silicate cements, is very low, possibly because of their method of measurement.

The thermal conductivities of dentin and enamel by Soyenkoff and Okun[4] were in reasonable agreement with the corresponding values reported in this study. The difference probably represents variations in procedure and sample preparation, particularly in the case of enamel. It should be noted that values for porcelain vary from

TABLE 4

COMPARISON OF THERMAL CONDUCTIVITIES WITH THOSE REPORTED IN THE LITERATURE

Material	Thermal Conductivity (Cal/Sec/Cm²/° C/Cm)	Temperature (° C.)	Source
Dentin	2.29×10^{-3}	40–90	Lisanti and Zander (Ref. 1)
Dentin	2.35×10^{-3}	.....	Simeral (Ref. 2)
Dentin	0.257×10^{-3}	.....	Phillips and co-workers (Ref. 5)
Dentin (root, ‖ to tubules)	0.96×10^{-3}	29	Soyenkoff and Okun (Ref. 4)
Dentin (crown, ‖ to tubules)	1.07×10^{-3}	29	Soyenkoff and Okun (Ref. 4)
Dentin (‖ to tubules)	1.36×10^{-3}	50	Craig and Peyton
Dentin (⊥ to tubules)	1.39×10^{-3}	50	Craig and Peyton
Enamel (‖ to rods)	1.55×10^{-3}	26–29	Soyenkoff and Okun (Ref. 4)
Enamel (∠ to rods)	1.56×10^{-3}	26–29	Soyenkoff and Okun (Ref. 4)
Enamel (‖ to rods)	2.23×10^{-3}	50	Craig and Peyton
Zinc phosphate cement	2.81×10^{-3}	.....	Simeral (Ref. 2)
Zinc phosphate cement	$3.91–5.39 \times 10^{-3}$	.....	Phillips and co-workers (Ref. 3)
Zinc phosphate cement (dry)	0.31×10^{-3}	.....	Phillips and co-workers (Ref. 5)
Zinc phosphate cement (wet)	0.35×10^{-3}	.....	Phillips and co-workers (Ref. 5)
Zinc phosphate cement (inlay mix)	2.5×10^{-3}	55	Craig and Peyton
Zinc phosphate cement (base mix)	3.1×10^{-3}	55	Craig and Peyton
Silicate cement	2.00×10^{-3}	.....	Simeral (Ref. 2)
Silicate cement	0.46×10^{-3}	.....	Phillips and co-workers (Ref. 5)
Silicate cement	$1.78–1.86 \times 10^{-3}$	45	Craig and Peyton
Dental amalgam	5.4×10^{-2}	36	Craig and Peyton

1.9 to 4.7×10^{-3}, with most of the values near 2.5×10^{-3} c.g.s. units. These values are close to those reported for dentin and enamel. The lower thermal conductivity of dentin compared with enamel is possibly a reflection of the larger amount of organic matrix present in the dentin.

The thermal conductivities of zinc phosphate and silicate cements reported by Simeral[2] of 2.81×10^{-3} and 2.00×10^{-3} c.g.s. units, respectively, were in good agreement with the values of $2.5–3.1 \times 10^{-3}$ and $1.78–1.86 \times 10^{-3}$ c.g.s. units found in this investigation. The early figures reported by Phillips[3] for zinc phosphate cement were in reasonable agreement with the previous values, but a later paper[5] lists the thermal conductivity as $0.31–0.35 \times 10^{-3}$ c.g.s. units, or about one-tenth the early values. Likewise, the value reported by Phillips[5] of 0.46×10^{-3} for silicate cement is much lower than those reported by the other authors.

The thermal conductivity of dental amalgam cannot be compared because of lack of literature values. It is interesting, however, that an amalgam containing possibly 45 per cent mercury, 39 per cent silver, 14 per cent tin, and 2 per cent copper, which

have individual conductivities of 0.019, 1.00, 0.14, and 0.92 cal/sec/cm^2/°C/cm, respectively, has a thermal conductivity of 0.054 c.g.s. units. The presence of mercury or amalgams, therefore, has a pronounced effect on the thermal conductivity of a finished amalgam restoration. The conductivity of the amalgam is only three times that of mercury, which is a relatively poor metallic thermal conductor.

The thermal conductivity of dental amalgam, therefore, is low compared with other restorative dental materials such as pure gold, 90 Au–10 Cu, and 80 Au–20 Cu, which have thermal conductivities of approximately 0.74, 0.29, and 0.17 cal/sec/cm^2/°C/cm, respectively. The conductivity of dental amalgam still is high compared with zinc phosphate and silicate cements, which have conductivities comparable to human tooth structure.

Thus it may be concluded that zinc phosphate and silicate cements are adequate thermal insulators in replacing lost tooth tissue. It also appears that a zinc phosphate cement base essentially replaces dentin in a deep-cavity preparation with respect to thermal insulation of the dental pulp. Although amalgams have considerably lower thermal conductivities than dental golds, a cement base would still be recommended in a deep-cavity preparation.

It should be emphasized that the numerical thermal conductivity values are independent of sample thickness, but the effectiveness of a cement base as an insulating medium is directly proportional to the thickness of the base. This fact should be considered in the placement of a cement base.

SUMMARY

The thermal conductivity of human dentin, enamel, zinc phosphate cement, silicate cement, and dental amalgram has been determined by using an improved steady-state procedure.

The average thermal conductivity of dentin was $1.36–1.39 \times 10^{-3}$ cal/sec/cm^2/°C/cm, which was lower than the value of 2.23×10^{-3} for human enamel.

Zinc phosphate and silicate cements had conductivities of $2.5–3.1 \times 10^{-3}$ and $1.78–1.86 \times 10^{-3}$ cal/sec/cm^2/°C/cm, respectively. These values approximate the value for tooth structure and are in the range of good thermal insulators.

Dental amalgam had an average conductivity of 5.4×10^{-2} cal/sec/cm^2/°C/cm, which places it as a relatively poor insulator compared with tooth structure or dental cements, but a relatively good insulator compared with dental gold alloys.

The authors gratefully acknowledge the assistance of I. Ibrahim, D. W. Johnson, R. G. Smith, and T. F. Peyton in the collection of the data.

REFERENCES

1. Lisanti, V. F., and Zander, H. A. Thermal Conductivity of Dentin, *J. D. Res.*, **29**:493, 1950.
2. Simeral, W. G. Thermal Conductivity of Dental Materials, Microfilm of papers presented at the Annual I.A.D.R. Dental Materials Group Meeting, March 17–18, 1951, *J. D. Res.*, **30**:499, 1951 (abstr.).
3. Phillips, R. W., Reinking, R. H., and Phillips, L. J. Thermal Conductivity of Dental Cement, *J. D. Res*, **33**:511, 1954.
4. Soyenkoff, B. C., and Okun, J. H. Thermal Conductivity Measurements of Dental Tissues with the Aid of Thermistors, *J.A.D.A.*, **57**:23, 1958.
5. Phillips, R. W., Johnson, R. J., and Phillips, L. J. An Improved Method for Measuring the Coefficient of Thermal Conductivity of Dental Cement, *J.A.D.A.*, **53**:577, 1956.

A Study of Posterior Mandibular Movements from Intercuspal Occlusal Position

WILLIAM L. KYDD and ALLAN SANDER
School of Dentistry, University of Washington, Seattle

Studies published in recent years on the positional relation of the mandible to the skull and movements of the mandible have left some questions unresolved. Basic differences of opinion exist. One group[1–6] contends that the occlusal or intercuspal position corresponds in normal dentition to the most retruded position or apex of the arrow-point tracing. Other investigators[7–12] have found evidence that a retrusive movement of 0.5–1 mm. from the median occlusal position or intercuspal occlusal position is a normal relationship between temporal bone and the mandible. These same investigators have found, in a small percentage of patients, that this retrusive movement may be absent.

The purpose of this investigation was to record posterior movements of the mandible occurring during (1) normal opening and closure, (2) during the swallowing cycle, (3) with and without the use of a suitable retractor. The existence of retrusive movement from the intercuspal occlusal position during swallowing has been hypothesized in persons with clinically normal occlusion; however, neither the frequency with which this movement exists nor the vector of this movement has been defined.

MATERIALS AND METHODS

A rapid film changer* was coupled with a 100 KV, 500 ma X-ray generator and an X-ray tube to provide serial 10 × 12 inch film of mandibular movement.[13] A mercury-vapor water-cooled contactor was employed to energize the X-ray generator.

The film changer and X-ray head were mounted on individual carriages on a table which was specifically designed to provide a variable and direct reading relationship between the central ray emitted by the X-ray anode and the center of the film. The X-ray head and film-changer carriages were interconnected by a steel tape calibrated to provide direct reading of film-target distance. Both carriages were movable in a direction parallel and at a 90° angle to the table. The latter direction was also calibrated on steel tapes, so that the central ray could be accurately centered on any point on the film in a horizontal plane. The X-ray head could also be moved vertically, with its movement calibrated on a steel tape to the center of the film in the vertical plane; hence the operator was able to position the central ray accurately on any point of the patient's head placed before the film.

A cephalostat for immobilizing and positioning of the patient's head was designed

This investigation was supported in part by U.S.P.H.S. research grant D-450 from the National Institute of Dental Research, National Institutes of Health.

Received for publication May 16, 1960.

* Schonander-Elema, Stockholm.

for direct mounting on the film changer (Figs. 1 and 2). The cephalostat was constructed of lucite rods for external auditory meatus supports, a nasion support, and an infraorbital ridge marker. The vertical lucite external auditory meatus rods were mounted on stainless-steel bars, which were calibrated in the horizontal plane to the center of the film. The rods were also calibrated vertically to the center of the film; hence, with the horizontal and vertical adjustments, the patient's external auditory meatus could be oriented to any point on the film. The infraorbital ridge marker was calibrated to the same vertical scale as the external auditory meatus supports; hence, by adjusting each scale to the same reading, the patient's Frankfurt plane could be oriented to the horizontal. The nasion support had an arbitrary vertical scale which could be used along with the other scales on the cephalostat for accurately repositioning patients for subsequent radiography. The horizontal scale for the nasion support provided the distance in centimeters from the nasion to the center of the film and also recorded the distance from the nasion to the superior surface of the external auditory meatus. The scale on the horizontal bar extending normal to the plane of the film was calibrated in centimeters from the film surface. For sagittal exposure the scale provided the subject-film distance (mid-sagittal plane-film distance) as well as the cephalic measurement (CM) for the patient.

Fourteen subjects were used for this investigation. The age group was between twenty and thirty-six years. Only persons having clinically good occlusions were used.

Tracing of mandibular movement was accomplished by following the movement of radio-opaque pellets. A lead pellet approximately 1.5 mm. in diameter was placed on the mandibular incisors and one pellet placed between the mandibuar first molar and second bicuspid. Placement of the anterior pellet was such that it would not interfere with occlusion. Reference pellets were placed on the maxillary teeth, one between central incisors and one between the first molar and second bicuspid. An auxiliary reference pellet was taped to the subject's forehead; however, since this pellet was attached to soft tissue, care was exercised in using this pellet as a reference point. All pellets were attached to the teeth with gray compound and on the quadrant adjacent to the film.[13]

Each subject was submitted to two serial X-rays: (1) one series of 16 pictures for an open-close-swallow tracing and (2) one series of 8 pictures for an open-close with retraction tracing.

The subject's head was placed in the cephalostat with the Frankfurt plane oriented to the horizontal. The superior surface of the external auditory meatus was placed at the center of the film, with the central ray oriented to pass through the center of the film. For the open-close-swallow series the subject was instructed to relax and move the mandible from occlusal position to full open and then close to occlusal position followed by a normal swallow. For the open-close with retraction series the subject was instructed to relax and "open and close the mouth." Following the open-close-swallow series, patients were retained in the cephalostat with identical nasion support, external auditory meatus support, and central-ray reading for the open-close with retraction series. Retraction of 3–4 lb. on the mandible was accomplished with the use of an elastic chin strap, with the force applied from the gnathion to the external meatus supports.

An intensifying screen and high-speed Lysholm vertical grid were used on all series. To minimize subject's enlargement, a target-film distance of 60 inches was used.

Allowing for differences in patient cephalic measurement, the average KV and ma was 86 and 320 at 1/30-second exposure. In order to be certain that all movement of the mandible is stopped in each picture, the preferred technique is 90 KV and 450 ma at 1/60-second exposure; however, because of X-ray generator limitations, it was necessary to use the lower KV and ma at the longer exposure time. Even at 1/30-second exposure, however, only in isolated cases when patient mandibular movement was very rapid could some movement of the pellets be detected on the film. All series were taken at 5 pictures per second, which allowed approximately 3 seconds for the open-close-swallow series and 2 seconds for the open-close with retraction series.

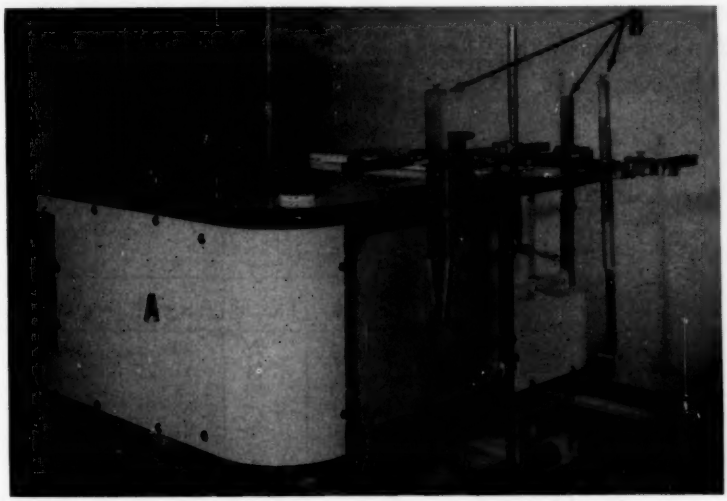


FIG. 1.—Cephalostat (*B*) mounted on rapid cassette changer (*A*)

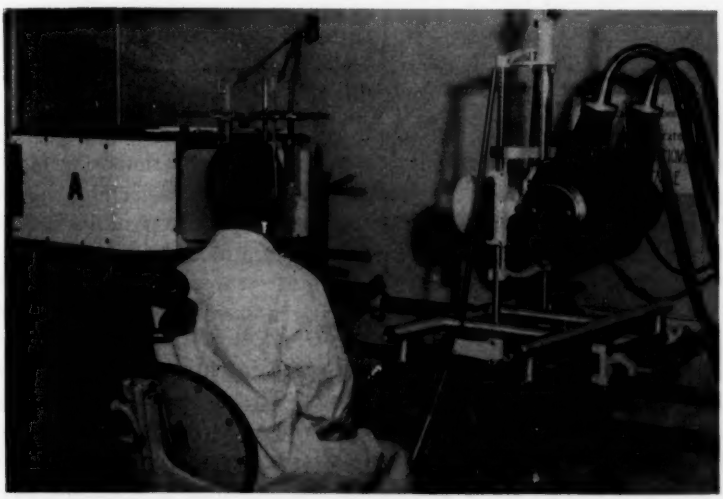


FIG. 2.—Subject oriented in cephalostat (*B*). Rapid film or cassette changer (*A*) and X-ray head shown mounted on individual calibrated carriages.

The processed film from the two series from each patient was compared visually over an X-ray viewbox to note superimposition of reference pellets. The reference pellets from each series were identical. The center of each pellet outline on each film was perforated by a sharp-pointed instrument. In this perforating procedure greater accuracy was obtained by using a Bausch & Lomb measuring magnifier. The position of the mandibular pellets on each film of each series was transferred to transparent orthodontic tracing paper. This was accomplished by superimposing the tracing paper over the film and perforating the tracing paper where the film had previously been perforated. Therefore, the outline of mandibular movement and corresponding condylar movement could be traced. By superimposing tracings of the open-close-swallow series and the open-close with retraction series, it was possible to compare the retrusion that occurred on swallowing with that which occurred with the retractor.

TABLE 1

VECTORS OF RETRUSION

Subject	Retrusion from Intercuspal Position (Mm.) Method Used		Angle of Retrusion to			Angle of Mean Condylar Path		
	Retractor	Swallowing	SN/P	O/P	FKT/P*	SN/P	O/P	FKT/P*
1.....	0.5	0.4	15	16	22	30	31	37
2.....	0.2	0.5	18	10	14	37	33	35
3.....	1.0	1.0	15	13	19	25	23	29
4.....	0.3	0.3	−34	−41	−28	36	29	42
5.....	0.5	0.5	14	11	18	34	31	38
6.....	0.5	0.3	28	21	25	28	21	25
7.....	0.2	0.2	15	10	12	28	16	24
8.....	0.9	0.9	27	19	26	27	19	26
9.....	1.8	0.6	−38	−47	−42	29	20	25
10.....	1.5	1.4	−46	−58	−52	44	32	38
11.....	1.1	1.1	−14	−12	−11	22	24	25
12.....	1.2	1.1	−17	−33	−20	36	20	33
13.....	1.7	1.4	34	22	30	33	21	29
14.....	0.9	0.8	−22	−28	−28	30	24	24

* SN/P = sella-nasion plane; O/P = occlusal plane; FKT/P = Frankfurt plane.

In addition, a second tracing procedure was used where a film on a subject illustrating maximal opening was made into a composite by superimposing films showing progressive degrees of closure to intercuspal position. The master film showed the sequential progress of the lead pellet to closure. In addition, the posterior movement was illustrated on the same film. All measurements were made with a Bausch & Lomb measuring magnifier.

RESULTS

Table 1 shows the vectors of retrusion observed in patients when swallowing and when the mandible was under a retracting force. The mean posterior movement with retraction was 0.87 ± 1.0 mm.; during swallowing, 0.71 ± 0.7 mm. All the patients displayed some posterior movement from their intercuspal position with either the retractor or when swallowing. Disregarding the direction of retrusion, the amount varied from 0.2 to 1.8 mm., and 6 cases displayed the same amount of retrusion during

swallowing as occurred forcibly under retraction. Using Student's *t*-test, no significant difference could be found between posterior movement during swallowing and the posterior movement with retraction. Cases that showed retrusion with the retractor but little on swallowing displayed comparatively deep interlocking cusps, such that the mandible was locked in intercuspal position during the swallowing cycle.

The direction of posterior movement was measured with respect to three standard planes, i.e., the sella-nasion plane, Frankfurt plane, and the occlusal plane (Fig. 3). Approximately half the cases examined showed positive angles of posterior movement with the occlusal plane or a direction of retrusion from intercuspal occlusal position approximating the mean condylar path. The remaining cases showed negative angles

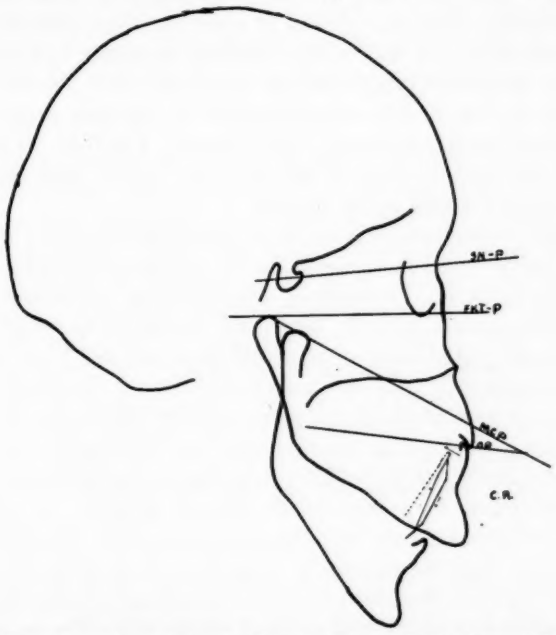


FIG. 3.—Illustrating sella-nasion plane, Frankfurt plane, occlusal plane. *MCP* = mean condylar path. Dotted line indicates path of mandibular opening and closure with retraction. Solid line denotes opening and closure with no encumbrance on the mandible. Arrows indicate direction of mandibular movement, i.e., opening and closing.

of retrusion. Examination of these cases revealed comparatively steep cusps, and consequently the vector of posterior movement was governed primarily by cuspal planes, producing posterior and inferior displacement of the condyles. The mean angle of retrusion in the positive direction was 17°, while the mean angle retrusion in the negative direction was —36°.

The condylar path from closed to open position was also measured with respect to three standard planes, i.e., sella-nasion plane, Frankfurt plane, and occlusal plane. The average angle of the condylar path to the occlusal plane was 25°.

The sagittal serial radiographs in the retracted group were examined for location of the transverse rotational plane of the mandible. In the retraction series, eight of the fourteen produced movement at the mandibular incisors approximating hinge-axis opening within the condyles. Rotational movement without translation occurred at

opening of 20 ± 3.1 mm. to closure at the incisors. In the remainder, rotational movement occurred, but its axis did not appear to be in the area of the condyles. Although mandibular retraction was used in all cases, the movement arc after an opening of 20 ± 3.1 mm. on the series showed an increase radius and an inferior displacement of the plane of rotation. This, of course, implies that while rotation occurred in space about the condyle the mandible moved bodily anteriorly and inferiorly.

DISCUSSION

Condylar registration in sagittal or profile radiographs presented the problem of overlapping dense bony structures which tend to obscure the bony anatomy of the temporomandibular joint, according to Amer.[14] Moreover, an examination of condyles in laboratory specimens will show shapes of great variation, presenting involute and evolute curvatures, which are not readily traceable in profile radiographs. Therefore, while mandibular movement can be defined accurately with the use of radio-opaque pellets, the outline of the condyle and registration of the mean condylar path passing through its geometric center can be only approximated. This does not imply that hinge-axis rotation did not occur in some of the subjects. It means that the axis of rotation did not appear within the area of the condyle.

The radiographic technique employed gave reasonable definition of deep soft-tissue outlines on the swallow series radiographs, and it was possible to some degree to follow muscular action in the series. Tremendous reflex muscular action and propulsive force occurred during the swallowing cycle, which was translated to retrusive movement of the mandible and substantial force to the dental structures and joint. Maximum retraction occurred simultaneously with maximum activity of the tongue and palatal musculature, giving rise to elevation of the soft palate and forward movement of the posterior wall of the pharynx, closing off the nasopharynx from the oropharynx. The swallowing cycle implies that a distinct retrusive force is applied to the mandible.

Spontaneous retrusion from intercuspal occlusal position occurring during the swallowing cycle coincided closely with the amount observed with forced retrusion. It is unlikely, therefore, that the supportive structures of the temporomandibular joint are noticeably compressible and that a retrusive position which may be observed under mandibular retraction is a forced and strained relationship. The amount of retrusion observed in the serial radiographs by voluntary action and under mandibular retraction agrees closely with that found by Posselt,[10] using graphic tracings. From the results obtained, the intercuspal occlusal position does not represent the most retruded position of the mandible. Also, as was demonstrated by Posselt, cases which we examined did not indicate a rest position within centric relation. The rest position did not appear to be a border position. We could not consistently demonstrate a rotation of the mandible with hinge axis within the area of the condyle with the use of a retracting force. The mandible in five subjects of the series demonstrated translatory motion immediately on opening, although under retraction the degree of translation was much less than that occurring during habitual opening. In those cases approximating rotational motion in opening, with the mandible under retraction, the patterns showed a marked degree of translation at openings at the incisors varying from 17 to 23 mm. Many authors have suggested that this change in motion is due to limitation in motion placed on the mandible by the temporomandibular ligament, i.e., at a certain range of opening, this ligament, because of its length, forces the mandible to move forward. We

do not propose to support or contradict this view, since an examination of a large number of prepared specimens and cases in vivo would be necessary to determine whether such limitation actually existed. Cases which we observed with the mandible under retraction generally showed the condyles in a more inferior position, suggesting that, while the posterior movement is limited by capsular structures, stability is primarily derived from the musculature. During swallowing, when muscle contraction may be considered unbalanced, stability is derived from the teeth or joint or both.

While all the cases examined demonstrated retrusion, considerable variance was displayed in the direction of retrusion. Approximately half demonstrated retrusion posteriorly and inferiorly, while the other half moved posteriorly and superiorly with a wide gradation in between. Lateral vectors of retrusion may also be present; however, these could not be demonstrated in sagittal view. The direction of retrusion is purely a function of existing cuspal planes, and the joint structures do not appear to influence the retrusive vector except to determine the posterior border position.

SUMMARY

All subjects demonstrated posterior movement from their intercuspal occlusal position. The retrusion varies from 0.2 to 1.8 mm. Considerable variance was observed in the direction of posterior movement. The direction of retrusion appears to be dependent primarily on cuspal planes rather than on the articulating surfaces of the temporomandibular joint. Retrusion obtained by the use of retraction was generally the same as that obtained in voluntary movement; therefore, it does not appear that temporomandibular joint structures are noticeably compressible from forceful retrusion. Rotary motion could not be consistently demonstrated with or without the mandible under retraction. The retractor is, however, effective in producing the posterior border position, but, once motion begins, some translation occurs. This study appears to indicate that the rest position does not represent a true border position. The intercuspal position is anterior to the most retruded position demonstrated in this study.

REFERENCES

1. McCollum, B. B. Fundamentals Involved in Prescribing Restorative Dental Remedies, *D. Items of Interest*, **61**:522, 641, 724, 852, 942, 1939.
2. Stuart, G. E. Articulation of Human Teeth, *D. Items of Interest*, **61**:1029, 1147; **62**:8, 106, 1939–40.
3. McLean, D. W. Diagnosis and Correction of Pathologic Occlusion, *J.A.D.A.*, **29**:1202, 1942.
4. ———. Pathologic Occlusion, a Major Clinical Problem, *ibid.*, **31**:1587, 1944.
5. Furnas, I. L. Problem of Establishing Centric Relation: Its Importance and Solution, *J.A.D.A.*, **22**:89, 1935.
6. ———. Centric Relation in Full Denture Construction, *ibid.*, **25**:718, 1938.
7. Sicher, H. Positions and Movements of the Mandible, *J.A.D.A.*, **48**:620, 1954.
8. Denen, H. E. Movements and Positional Relations of the Mandible, *J.A.D.A.*, **25**:548, 1938.
9. Edmand, P. A. Restoring Lost Vertical Dimension, *J.A.D.A.*, **25**:849, 1938.
10. Posselt, U. Studies in the Mobility of the Human Mandible, *Acta Odont. Scand.*, Vol. **10.** Suppl. 10, pp. 13–160, 1952.
11. Posselt, U., and Yahr, R. G. A Comparison of the Gnatho-thesiometer with Lateral Cephalometric and Temporomandibular Joint Radiographs in Measuring Certain Antero-posterior Positions of the Mandible, *Odont. Revy*, **8**:458, 1957.
12. Boos, R. H. Intermaxillary Relationship Established by Biting Power, *J.A.D.A.*, **27**:1192, 1940.
13. Kydd, W. L. Rapid Serial Roentgenographic Cephalometry for Observing Mandibular Movements, *J. Pros. Den.*, **8**:880, 1952.
14. Amer, Ayoub. Approach to Surgical Diagnosis of the Temporomandibular Articulation through Basic Studies of the Normal, *J.A.D.A.*, **45**:668, 1952.

Experimental Carcinogenesis in Submandibular Glands of Hamsters

A. P. CHAUDHRY, D. H. REYNOLDS, R. J. GORLIN, and R. A. VICKERS
School of Dentistry, University of Minnesota, Minneapolis

The literature concerning experimental induction of tumors in salivary glands of animals has been recently reviewed by Standish.[1] Steiner[2] conducted the first well-planned investigation into the comparative pathology of induced tumors of the salivary glands in different animals. By employing hydrocarbon carcinogens like methylcholanthrene, 1,2,5,6-dibenzanthracene, and 3,4,-benzpyrene in the form of pellets, he was able to induce metaplasia of the glandular epithelium, epidermoid cysts, squamous-cell carcinoma, and adenocarcinoma. He found that methylcholanthrene produced more sarcomas and a greater variety of mixed tumors in mice than did dibenzanthracene. Bauer and Byrne,[3] using the same type of carcinogens and the same method of administration, were also able to produce epithelial malignant tumors. Standish studied histologically the evolvement of these tumors. His findings were similar to those of Steiner. He also noted that methylcholanthrene induced sarcomas more frequently than did 7:12 dimethylbenz(α)anthracene.

The present investigation was undertaken to study the effects of a single injection of a hydrocarbon carcinogen on the different tissue components of salivary glands and the subsequent tumor formation. Furthermore, it is to be used as a tool for future investigations for the understanding of some of the intricate mechanisms underlying cancer in general. The experimental analysis was concerned with (1) the histopathologic changes in the submandibular glands at certain slated time intervals following a single injection of a hydrocarbon carcinogen; (2) the effects of different concentrations of a carcinogen injection on the number of tumors induced; and (3) the effects of the animal age on the tumor yield.

MATERIALS AND METHODS

First, 50 golden Syrian male hamsters, 3 months of age, were divided into six groups. The animals were singly housed in a room maintained at 72° ± 3° F., illuminated from 6:00 A.M. to 6:00 P.M. and darkened from 6:00 P.M. to 6:00 A.M. Fox chow and tap water were available ad libitum. Under intraperitoneal nembutal anesthesia, a vertical incision was made in the middle of the ventral surface of the neck. By blunt dissection, submaxillary glands were exposed, and 0.05 cc. of 0.5 per cent 7:12 dimethylbenz(α)anthracene dissolved in liquid petrolatum was injected into the right gland. The left gland was injected only with the solvent in the same amount and served as control. The animals from groups I through VI were sacrificed with lethal doses of

This study was supported by research grant 350-8658-2800 from the American Cancer Society, Minnesota Section.

Received for publication June 8, 1960.

nembutal at intervals of 1, 4, 8, 10, 14, and 18 weeks, respectively. Immediately after extirpation, the glands were fixed in Zenker's solution for 24 hours. They were imbedded in paraffin and processed for microscopic sections in routine fashion. They were stained with hematoxylin and eosin by the quick method described by Clark and Gorlin.[4]

Another 45 golden Syrian male hamsters, 3 months of age, were divided into three groups and were kept under conditions similar to those in the preceding experiment. The right submaxillary gland of each animal in group I was injected with 0.05 cc. of 0.1 per cent 7:12 dimethylbenz(α)anthracene. The animals from groups II and III were injected with the same amount of carcinogen in concentrations of 0.25 and 1.0 per cent, respectively. The left gland was treated the same as in experiment I and served as control. The animals were sacrificed in the same fashion after 18 weeks. The submaxillary glands were examined microscopically, and the number of animals with tumors was recorded.

In a third study, three groups of 10 golden Syrian male hamsters in each were used. They were 1, 6, and 9 months of age, respectively. As previously described, 0.05 cc. of 0.5 per cent solution of 7:12 dimethylbenz(α)anthracene was injected into the right submaxillary gland of each animal, and the same amount of solvent into the left gland. The animals were sacrificed at the end of 18 weeks, and the glands were processed in the same manner as previously described. The number of animals with tumors, confirmed by microscopic study, was recorded.

RESULTS

Histopathologic changes.—At 1-week intervals the carcinogen evoked minimal inflammatory reaction but had caused extensive degeneration of the tissue, both parenchymatous and stromal, in its immediate environs. The gland tissue in close proximity to the injection site was replaced by faintly staining eosinophilic material not unlike fibrin, but its exact nature could not be ascertained (Fig. 1). Throughout this material there were scattered remnants of nuclear material which stained deep blue but without any cellular detail. Some ducts and acini were still present in those parts of the gland distant from the injection site. The epithelial lining of these structures showed squamous metaplasia, and their lumina were filled with cellular debris and inflammatory cells (Fig. 2). At the fourth week, the eosinophilic material stained more deeply and presented a hyalinized appearance. Nuclear remnants were slightly more prominent. The inflammatory reaction was easily discernible, and in a few instances there was abscess formation. At the eighth week, in addition to the above changes, there was evidence of some fibroblastic activity throughout the eosinophilic material. The fibroblasts were arranged in disordered fashion. They were not of uniform size and shape, and their nuclei appeared slightly hyperchromatic. At the tenth week, quite a few atypical cells with a large cytoplasm and a big nucleus had appeared and were suggestive of their malignant nature (Fig. 3). Three of the 9 animals in this group had developed fibrosarcomas of varying gradations at the end of the fourteenth week. In well-differentiated fibrosarcomas the cells were pleomorphic, with a moderate quantity of cytoplasm and a relatively large hyperchromatic nucleus containing one or more prominent nucleoli (Fig. 4). Mitotic activity was frequently seen. In poorly differentiated sarcomas there was an extensive degree of pleomorphism. The cells were unusual-

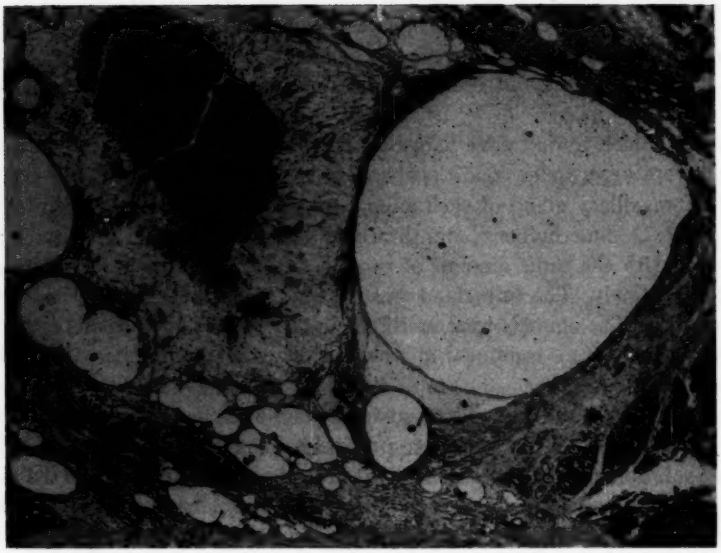


FIG. 1.—Photomicrograph, demonstrating histologic changes 1 week following injection of carcinogen. Note replacement of parenchymatous tissue by light-staining material. Spaces indicate site of carcinogen deposition.

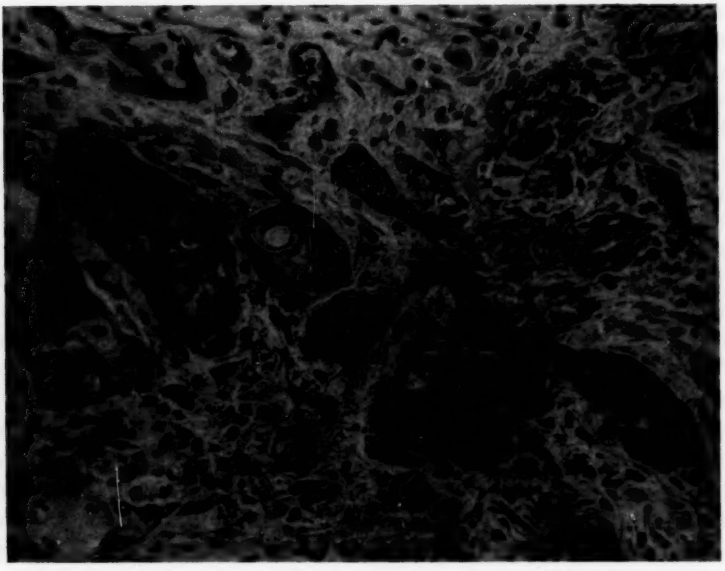


FIG. 2.—Photomicrograph, demonstrating hyperplasia and squamous metaplasia of parenchyma

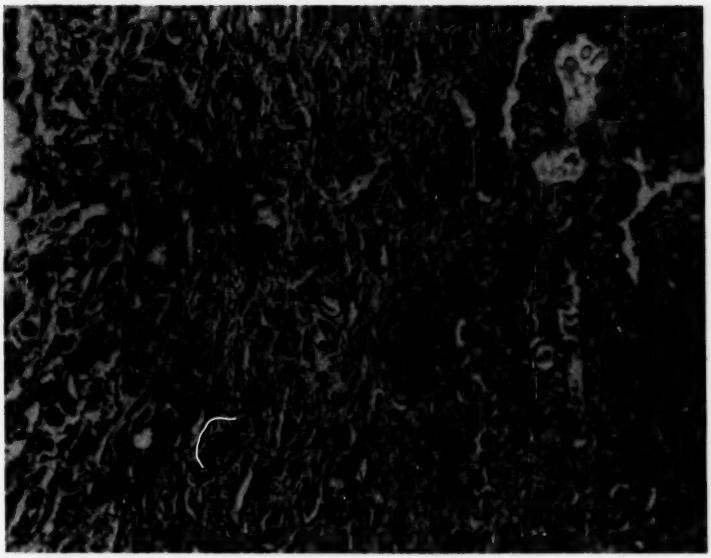


FIG. 3.—Photomicrograph, demonstrating histologic changes (10 weeks post-injection). Note hyperchromatism and irregularity of connective tissue cells.

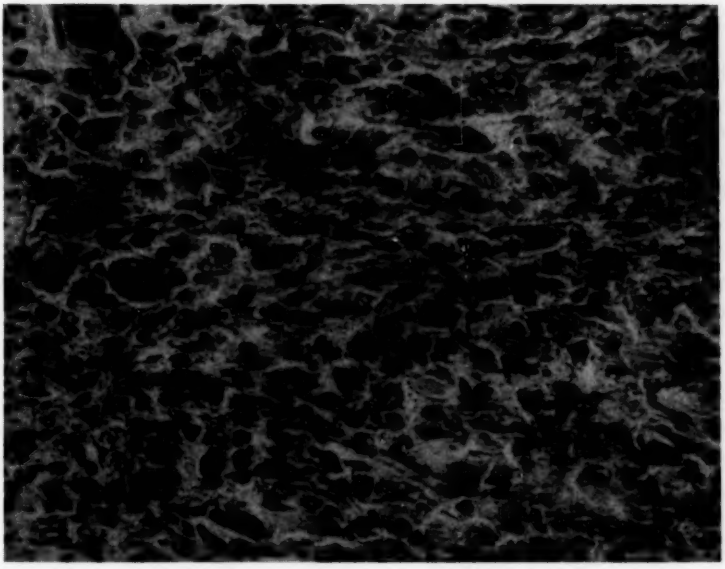


FIG. 4.—Photomicrograph, demonstrating poorly differentiated sarcoma (14 weeks post-injection). Note extreme hyperchromatism and pleomorphism with giant nucleated cell.

ly large, with giant nuclei. Mitotic activity was marked, and abnormal mitoses were quite common. Fibrosarcomatous appearance was still evident in parts of the tumors. The incidence of the tumor was higher and the differentiation poorer at the end of the eighteenth week. However, no local or distant metastases could be found up to this time.

In control animals no significant change was noticeable in different groups. The solvent had evoked minimal inflammatory reaction except for the abscess formation in occasional animals. Foreign-body giant cells were seen rarely, and neoplastic changes were completely absent.

The results of experiment II are shown in Table 1.* It is quite evident that, with an increase in concentration of carcinogen, the number of tumors is also increased. The systematic intergroup difference in the tumor yield was statistically significant at the 0.005 level. Figure 5 is the graphic representation of the dose-response curve. The

TABLE 1

EFFECTS OF DIFFERENT CONCENTRATIONS OF CARCINOGENS ON TUMOR INCIDENCE

EXPERIMENT	GROUP	AGE	QUANTITY INJECTED		ANIMALS EMPLOYED	ANIMALS WITH CANCER	PER CENT ANIMALS WITH CANCER
			Carcinogen	Solution			
I.....	VI	3 months	250 μg.	0.05 cc.	9	4	44.5
II.....	I	3 months	50 μg.	0.05 cc.	13	1	7.7
	II	3 months	125 μg.	0.05 cc.	16	4	25.0
	III	3 months	500 μg.	0.05 cc.	13	7	55.4

number in parentheses indicates the number of animals used at each dose. The plotted points represent on the vertical scale the actual proportion of the animals developing tumors with the dose shown on the horizontal scale. The vertical lines through each of the plotted points represent an interval of 1 SE on each side of the plotted percentage. The dark line is the least-squares line. The slope of the curve is highly significant of systematic intergroup difference in the dose response.

The results of the third study are shown in Table 2. It is obvious that, until 9 months of age, there is no difference in the incidence of tumor yield. In all three groups of animals sacrificed at the end of 18 weeks the total number of tumors was 44.5 per cent.

DISCUSSION

This is a preliminary report on the study of the effects of a single injection of a hydrocarbon carcinogen in the submaxillary glands of the hamsters kept under standardized environments. It was observed that a single injection of 0.05 cc. of 0.5 per cent (250 mg.) 7:12 dimethylbenz(α)anthracene dissolved in liquid petrolatum would induce a sarcoma in the glands between 10 and 14 weeks. At about 10 weeks, an alteration in the morphology of individual cells was noticeable, but it was not until 14 weeks that frank fibrosarcomas were produced in this study. There was a minimal response on the part of the epithelial components of the glands to the carcinogen. This was due to

* Group VI represents results of experiment I in this study.

the fact that the toxicity of the carcinogen destroyed the ducts and acini, which were replaced by scar tissue. It appeared that, by the further action of the carcinogen on the newly formed connective tissue, fibrosarcomas were produced. When the carcinogen is administered in the form of pellets,[2] its slow and gradual action on the more sensitive glandular portion produces such changes as metaplasia of the ductal and acinar

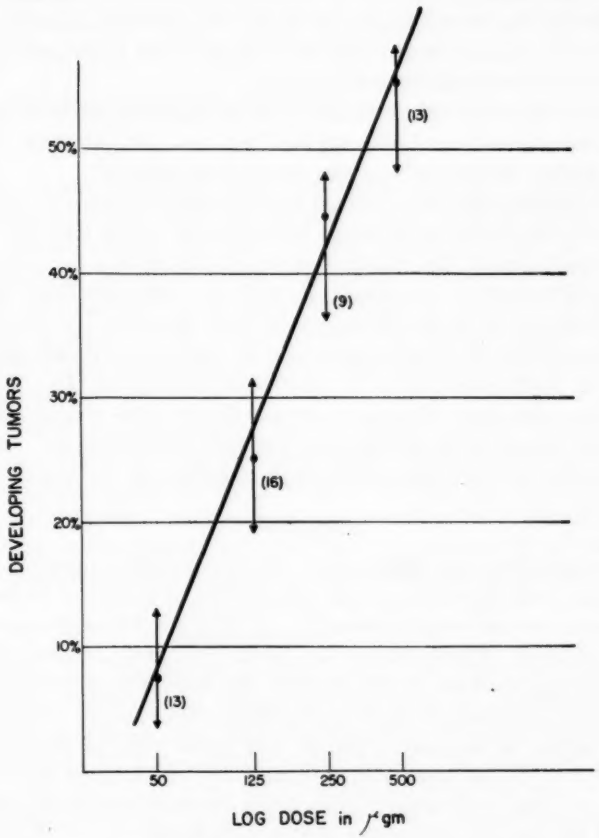


FIG. 5.—Graphic representation of dose-response curve

TABLE 2

EFFECT OF AGE ON TUMOR INCIDENCE

EXPERIMENT	GROUP	AGE	QUANTITY INJECTED		ANIMALS EMPLOYED	ANIMALS WITH CANCER	PER CENT ANIMALS WITH CANCER
			Carcinogen	Solution			
I.....	VI	3 months	250 μg.	0.05 cc.	9	4	44.5
II.....	I	1 month	250 μg.	0.05 cc.	9	4	44.5
	II	6 months	250 μg.	0.05 cc.	9	4	44.5
	III	9 months	250 μg.	0.05 cc.	9	4	44.5

epithelium, formation of epidermoid cysts, and eventually squamous-cell carcinoma, as has been repeatedly shown by Steiner,[2] Bauer,[2] and Standish.[1] Thus it would appear that mode of administration of 7:12 dimethylbenz(α)anthracene may be one of the factors in determining the types of the neoplasms that could be induced in the salivary glands. Other determining factors could be the agents used and their concentrations, the species, and the stock of the animals employed. Rusch, Baumann, and Marson,[5] for example, claimed to have induced squamous-cell carcinoma in mice of stock "A" by the injection of 3:4 benzpyrene dissolved in oil and given in amounts of 0.3–0.7 mg. These factors would need further investigations.

The tumor yield varied directly with the increase in concentration of the carcinogen. This factor, however, was tested only up to 1 per cent concentration. The effects of concentrations higher than 1 per cent are under investigation.

Age, up to 9 months, was not a critical factor in the incidence of induced tumors. However, it would be of interest to study the effects of age in hamsters older than 9 months. The importance of the standardization of the animals is apparent from the results that, in each group of 9 animals, 4 (44.5 per cent) developed sarcomas. The identical percentages in the three age groups as such, however, are embarrassing from a statistical point of view. It is not implied that in another study such percentages will again be identical. Differences are to be expected in replications. The implication may be made, however, that such differences are not likely to be statistically significant. This suggestion is supported by the fact that the slides were "blinded" and then examined microscopically by two investigators independently, on two occasions.

SUMMARY

When 0.05 cc. of 0.5 per cent (250 mg.) 1:12 dimethylbenz(α)anthracene dissolved in liquid petrolatum was injected into the submandibular glands of 3-month-old hamsters, fibrosarcomas were induced between 10 and 14 weeks. The sequence of microscopic changes were, in order, degeneration of the parenchymatous and stromal tissue of the gland in the immediate environs of the injection site, fibrosis, and its subsequent transformation into fibrosarcomas of varying gradations. No changes of any significance were, however, noticed in the control glands. The tumor yield at the end of 18 weeks increased from 7.7 to 55.4 per cent with the change in concentration from 0.1 (50 mg.) to 1.0 per cent (500 mg.). All other factors being constant, the age of the animals up to 9 months did not change the number of tumors induced.

REFERENCES

1. Standish, S. M. Early Histologic Changes in Induced Tumors of the Submaxillary Salivary Glands of the Rat, *Am. J. Pathol.*, **33**:671, 1957.
2. Steiner, P. E. Comparative Pathology of Induced Tumors of the Salivary Glands, *Arch. Pathol.*, **34**:613, 1942.
3. Bauer, W. H., and Byrne, J. J. Induced Tumors of the Parotid Gland, *Cancer Research*, **10**:755, 1950.
4. Clark, H., and Gorlin, R. J. On the Use of a Modified Fifteen-Minute Hematoxylin and Eosin Tissue Stain, *Oral Surg., Oral Med. Oral Path.*, **11**:431, 1958.
5. Rusch, H. P., Baumann, C. A., and Masson, G. L. Production of Internal Tumors with Chemical Carcinogens, *Arch. Pathol.*, **29**:8, 1940.

Amount, pH, Buffer Effect, and Amylolytic Activity of Parotid Saliva between and Immediately Following Meals

*BO KRASSE**

University of Alabama, School of Dentistry, Birmingham

Saliva stimulated immediately following an ordinary meal by chewing paraffin wax or chewing gum shows a significantly higher buffer effect than saliva secreted after the same stimulus between meals, and the difference seems to be due to the bicarbonate content.[1] An increased buffer effect after meals was also reported by Ericsson.[2] These findings, which were made on mixed saliva, could possibly be explained by variations in the proportional amounts of saliva stimulated from various glands,[3] as it has been found that parotid and submaxillary salivas show considerable variations in electrolytic concentrations.[4]

The aim of the present investigation was to study the effect of meal-taking on the rate of flow and composition of a separate salivary secretion produced in response to standard stimulation. In order to use chewing as stimulation, the parotid glands were selected for study. It seemed worthwhile to test not only the buffer effect but also the amylolytic activity of the parotid saliva, to find out whether the secretion of electrolytes and of organic material is influenced in different ways by the meal.

MATERIALS AND METHODS

Sixteen persons, four females and twelve males, twenty to thirty-five years of age, served as subjects. Parotid saliva was collected by means of parotid cups during exactly 5 minutes of chewing paraffin wax. The samples were taken between meals about 10:30 A.M. and about 12:30 immediately after lunch. The amount of saliva produced was measured. The space above the saliva in the test tube was filled with expired air, in order to get the same carbon dioxide tension as in the mouth. The pH was measured, and the buffer effect was determined by potentiometric titration with 0.01 normal hydrochloric acid. Amylase activity was determined by the method of Myers, Free, and Rosinski[5] and converted on the basis of the data of Schneyer[6] to milligrams of crystalline enzyme.

RESULTS

As shown in Table 1, the pH and the buffer effect were significantly higher in the parotid saliva stimulated at meals than in that produced by the same stimulus between the meals. An increased amount of saliva was also found at the meals, although here

Received for publication June 13, 1960.

* Present address: Royal Dental School, Malmö, Sweden.

the probability of difference is not so great as in the other cases. With regard to the amylase, no clear-cut change was observed. In eight persons there was an increase of the amylase content, in four there was a decrease, and in four the values were about the same between and at meals.

DISCUSSION

This study demonstrates that in parotid saliva the buffer effect is significantly higher immediately after a meal than between meals. No definite change was observed in the amylolytic activity, under the conditions of stimulation employed. This is in agreement with data previously reported by Schneyer.[6] It has been shown that the buffer

TABLE 1

AMOUNT, pH, BUFFER EFFECT, AND AMYLOLYTIC ACTIVITY OF PAROTID SALIVA STIMULATED BY CHEWING PARAFFIN WAX FOR 5 MINUTES BETWEEN AND IMMEDIATELY FOLLOWING MEALS

(Mean Values from 16 Individuals)

	Amount (Ml.)			pH			Buffer Effect*			Mg Amylase/Ml		
	Between Meals	At Meals†	Diff.	Between Meals	At Meals	Diff.	Between Meals	At Meals	Diff.	Between Meals	At Meals	Diff.
M (mean value)	4.05	5.46	+1.41	7.08	7.36	+0.28	13.4	19.4	+6.0	1.33	1.48	+0.15
m (standard error)	.....	.....	±0.42	.....	.....	±0.04	.....	.....	±0.9	.....	.....	±0.13
t	.....	.....	3.36	.....	.....	7.0	.....	.....	6.67	.....	.....	1.14
P	0.01>P>0.001			<0.001			<0.001			>0.1		

* Expressed as m Ekv HCl/liter to pH 5.0.

† Refers to period immediately following meal.

effect of saliva is largely dependent on the carbon dioxide content[7, 8] and that an increase in buffer effect in mixed saliva at meals is caused by an increase in the amount of bicarbonate.[1] Thus it is probable that the increased buffer effect in parotid saliva after a meal is also due to an increase in bicarbonate. This would mean that that part of the saliva secretion which consists of a passage of crystalloids from the capillary is influenced by the meal; i.e., the change in saliva should be of the same nature as the postprandial alkaline tide in urine. This assumption is supported by the observation of Anderson[9] that an increase in plasma bicarbonate after peroral administration of $NaHCO_3$ was accompanied by an increase in salivary pH.

On the other hand, Sand[10] and Ericsson[2] did not find any definite influence on bicarbonate content or buffering action of saliva after experimental alkalosis, and, according to Sand, bicarbonate is formed in the cells of the salivary glands during the secretion process. Takaoka[11] seems to be of the same opinion. But it is also known that experimental acidosis or alkalosis in dogs changes the bicarbonate and pH of the saliva[12] and that "saliva produced during or immediately after hyperventilation, which lowers the H_2CO_3 fraction of blood, has an increased pH."[13] If these conflicting views of the influence of variations in the plasma on the composition of saliva are to be solved, further studies using saliva collected from the separate salivary glands are necessary.

SUMMARY

The pH and buffer effect were found to be significantly higher in parotid saliva stimulated immediately after meals than in that produced by the same stimulus between the meals. A tendency toward an increased amount of saliva was found at meals, but with regard to amylolytic activity no clear-cut change was observed.

To Drs. J. F. Volker, L. H. Schneyer, C. A. Schneyer, and J. M. Bruhn I extend my sincere gratitude for stimulating discussions and for facilitation of this study.

REFERENCES

1. Krasse, B., and Gustafsson, B. Buffer Effect of Saliva Stimulated between and at Meals, *J.A.D.A.*, **56**:50, 1958.
2. Ericsson, Y. Clinical Investigations of the Salivary Buffering Action, *Acta Odont. Scand.*, **17**: 131, 1959.
3. Schneyer, L. H., and Lawrence, K. L. Rate of Secretion by Exogenously Stimulated Salivary Gland Pairs of Man, *J. Appl. Physiol.*, **7**:609, 1955.
4. Henriques, B. L., and Chauncy, H. H. Comparative Electrolytic Concentrations of Submaxillary and Parotid Salivas, *J. D. Res.*, **37**:28, 1958.
5. Myers, V. C., Free, A. H., and Rosinski, E. E. Studies on Animal Diastases. VI. The Determination of Diastase (Amylase) in Blood, *J. Biol. Chem.*, **154**:39, 1944.
6. Schneyer, L. H. Amylase Content of Separate Salivary Gland Secretions of Man, *J. Appl. Physiol.*, **9**:453, 1956.
7. Wah Leung, S. A Demonstration of the Importance of Bicarbonate as a Salivary Buffer, *J. D. Res.*, **30**:403, 1951.
8. Lilienthal, B. Analysis of the Buffer Systems in Saliva, *J. D. Res.*, **34**:516, 1955.
9. Anderson, D. J. The Relationship between Hydrogen-Ion Concentration and Rate of Flow of Saliva, *J. D. Res.*, **28**:583, 1949.
10. Sand, H. F. Source of the Bicarbonate of Saliva, *J. Appl. Physiol.*, **4**:66, 1951.
11. Takaoka, W. Acid Base Balance of Saliva. II. Essential Factors Governing the Acid Base Balance of Saliva, *Chem. Abstr.*, **41**:11443, 1953.
12. Benetato, G., and Opriusu, C. Variations in the pH and Bicarbonates of the Saliva in Provoked Acidosis and Alkalosis (Dog), *Chem. Abstr.*, **32**:6321, 1938.
13. West, E. S., and Todd, W. R. *Textbook of Biochemistry*. 2d ed. New York: Macmillian Co., 1957.

Histochemistry of Oral Mucous Membrane: Total Protein, Sulfhydryls, Disulfides, Ribonucleic Acid, and Desoxyribonucleic Acid

LOUIS A. CANCELLARO, JULES KLINGSBERG,† and EARL O. BUTCHER*

Departments of Anatomy, College of Dentistry and the Graduate School of Arts and Science, New York University, and the Hahnemann Medical College and Hospital of Philadelphia

Distribution in the oral mucous membranes of disulfide and sulfhydryl groups has been reported by several investigators.[1–4] The occurrence of ribonucleic and desoxyribonucleic acid has also been described and related to inflammation. The object of the present study is to compare the distribution of these substances in different areas of the oral cavity and to evaluate their significance in relation to aging and to keratinization processes in two commonly used laboratory animals—the rat and the hamster. These two rodents have been chosen because they have a short life-span and the entire history of their oral tissues can be studied in a relatively short interval. In addition, one animal—the hamster—is susceptible to periodontal disease while the other—the Long-Evans rat—is relatively resistant, even in advanced age.

MATERIALS AND METHODS

One hundred male Long-Evans rats and 75 male Syrian hamsters were sacrificed at various intervals from 1 to 900 days of age. The oldest hamsters were sacrificed at 450–540 days, since too many died after this age from natural causes. Four rectangular strips of gingiva, extending from the first to the third molar, including the marginal gingiva, were dissected from the buccal and lingual surfaces of the mandible. Four pieces of buccal and palatal gingivae were similarly removed from the upper jaw. This left a strip 2 mm. in width of palate mucous membrane in the mid-line. A rectangular piece of mucous membrane, designated as the "palate cheek specimen," was dissected from each cheek and the anterior portion of the palate. A total of eleven specimens was taken from each animal, and twenty-five serial sections were made from each. The rats and hamsters were sacrificed, either by an overdose of ether, by intraperitoneal injections of sodium nembutal, or by cranial trauma. No differences resulted in the tissues from the method of sacrificing. The tissues were fixed either in alcoholic formalin

---

Part of this investigation was presented to the Graduate School of Arts and Science, New York University, in partial fulfilment for the requirements for the degree of Doctor of Philosophy in the Department of Anatomy.

This study was supported by U.S.P.H.S. research grant D-366 from the National Institute of Dental Research, National Institutes of Health.

Received for publication June 20, 1960.

* Presently at the Hahnemann Medical College and Hospital, Philadelphia.

† Postdoctoral Research Fellow of the National Institute of Dental Research.

(AAF) (85 parts absolute alcohol, 5 parts glacial acetic acid, and 10 parts 40 per cent formaldehyde), trichloro-acetic acid (1 gm. trichloro-acetic acid dissolved in 100 cc. of 80 per cent ethanol), or 10 per cent formalin. No differences resulted in the staining reactions from the choice of fixatives. All tissues were fixed for 4 hours at 4° C. Paraffin with a melting point of 56°–58° C. was used for imbedding, and the tissues were sectioned at a thickness of 8 μ. Keratinization was evaluated in this study by Castenfelt's criterion, which is based on staining characteristics of cytoplasm, and by cellular and nuclear morphology. The following histochemical techniques were used.

The Feulgen periodic acid–Schiff technique[6] was used to demonstrate desoxyribonucleic acid. Control slides were incubated overnight at 37° C. in 0.002 per cent desoxyribonuclease buffered with Sorenson's to pH 6.5. Ribonucleic acid was demonstrated by using toluidine blue.[7] Control slides were incubated overnight at 37° C. in 0.1 per cent ribonuclease and buffered with Sorenson's to pH 6.5 or in buffer alone.

The hematoxylin method of Jarrett and Hardy[8] was employed as a confirmatory technique for the presence of ribonucleic and desoxyrbonucleic acids, since this procedure stains both acids, if present, in the tissue. Slides were incubated overnight at 37° C. with the 0.05 per cent ribonuclease, buffered with Sorenson's to pH 6.5, or with 0.002 per cent desoxyribonuclease, buffered with Sorenson's to pH 6.5, or in buffer alone.

The method of Barrnett and Seligman[10] was used for disulfide and sulfhydryl localizations. *N*-ethyl maleimide was used as a blocking agent. The method of Pearse,[11] which employs blue tetrazolium, was used as a confirmatory method for disulfide determination. The brom-phenol blue technique of Mazia and Brewer[12] was used for total protein determination.

RESULTS

Sulfhydryl and disulfides.—Utilization of the method of Barrnett and Seligman involved the improvisation of a gradated color scale which could be used to determine the degree of intensity of the localization elicited by the binding capacity of the dye for the protein group. The colors—pale pink, light (1); strong pink and pale blue, moderate (2); bluish pink, intense (3); and blue–bluish purple, very intense (4)—are dependent on the concentration of sulfhydryl and disulfide groups present in the tissue, or the tetrazolium monocoupled or dicoupled to the reacted napthol moieties of the reagent (Barrnett).[13, 14]

At 1 day of age (Fig. 1) in the rat, the sulfhydryl groups of the epithelium of the oral mucus membrane stain faintly in the lower layers and more intensely in the corneum. The staining of the stratum basale was more pronounced than the prickle-cell layer. Comparison of several sections stained by Feulgen Schiff with those stained in hematoxylin and eosin showed the stratum corneum to be parakeratotic (Fig. 2).

Between 1 and 8 days of age there appears to be an increase in reactive SH groups in the stratum corneum and Malpighii layer, but no extensive change was observed between the 8-day-old and 54-day-old rats (Fig. 3). A study of tissues from 800- to 900-day-old rats revealed no changes in the stratum Malpighii as compared with 8-day-old animals, but the outermost layers of the corneum decreased in staining intensity (Fig. 4). A comparison of 42-day-old hamsters with the 63-day-old rats revealed similar distribution of SH groups in the corneum and the stratum Malpighii. The 420-day-old

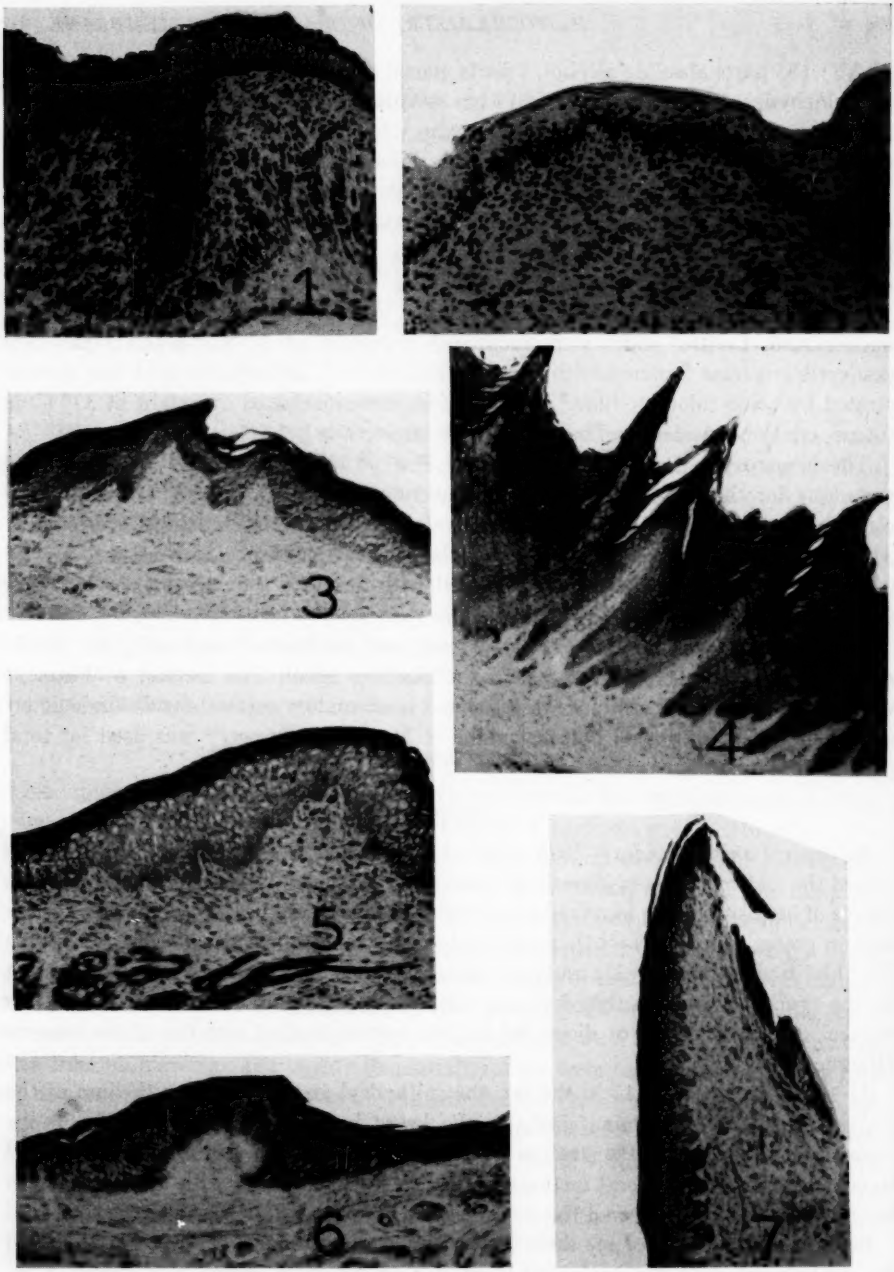


FIG. 1.—Upper jaw from 1-day-old rat shows distribution of postive sufhydryl groups in the parakeratotic zone of the stratum corneum, with a lesser reaction in stratum Malpighii. Note increase in intensity in stratum basale. This may be associated with the formation of the dental lamina. Barrnett and Seligman method. ×100.

FIG. 2.—Feulgen reaction for DNA in upper jaw of 1-day-old rat. Section taken from same animal as in Fig. 1. The persisting nuclei seen at the periphery are indicative of parakeratosis. ×100.

FIG. 3.—Sulfhydryl distribution in the palate of 63-day-old rat. Note increase in intensity as compared with 1 day. Barrnett and Seligman method. ×100.

(Legends continued on next page.)

hamster showed no change from the 42-day-old hamster. A study of the 7-day-old hamster revealed no significant differences in distribution of SH from that of the 42-day-old and 420-day-old hamsters.

An intense staining band, which is apparently similar to the keratogenous zone of Giroud in hard keratin, was found in the corneum of rats and hamsters, 42 days and older (Fig. 4). A similar staining reaction was evidenced in the outermost epithelial layer of the 1-day rat. The layer in the young rat is comparable to the outermost layer of the stratum Malpighii of the older animals.

At all ages studied, disulfides were found to be present throughout all layers of the epithelium, being more pronounced in the corneum than in the deeper strata. No differences were evident between rats and hamsters.

The nucleus, as well as the cytoplasm of the cells in the basal layer and stratum spinosum, contained granules, which appeared to be reactive for disulfide. A granular sulfhydryl reaction was also present in the nucleus, while the cytoplasm appeared homogenous.

A positive reaction for disulfides was observed for the intercellular material. In using the Barrnett and Seligman technique for combined SH and SS, a moderate reaction was noted in the lower layers of the stratum Malpighii and a more intense reaction in the corneum (Figs. 5, 6). These findings were for both the rat and the hamster. There were no alterations in the staining reaction in different parts of the oral mucous membrane (Fig. 7).

Pearse's blue tetrazolium technique was used as a confirmatory method for disulfides. Granules were found to be present in all the cells of the basal and spinosum layers, being more intensely deposited in the basal-cell layer. A weak disulfide reaction was found in the Malpighii layer, with intensification in the corneum (Figs. 8, 9). The staining reaction was similar in the various regons of the oral mucous membrane (Fig. 10).

The intercellular cement had a clearly positive reaction for both sulfhydryls and disulfides at all ages except in the 1-day-old animal, where this reaction was less distinct.

It was observed that the intercellular cement stained more distinctly for sulfhydryls and disulfides in the upper layers of the stratum spinosum than in the remaining layers of the stratum Malpighii.

Sulfhydryls were found to be present in the tunica propria of both rats and hamsters. Although the entire tunica propria of the older animals seemed to stain slightly darker than the younger, the difference was insufficient to warrant the conclusion that an age change existed. The undifferentiated (mesenchymal) cells of the 1-day-old animal demonstrated an exceedingly strong staining reaction (Fig. 1), while the fibroblasts in the older animals showed more variations.

The intercellular cement of the epithelium stained more darkly than the ground substance of the connective tissue. This indicates that there are fewer reactive sulfhydryl

---

FIG. 4.—Distribution of SH groups in palate of 850-day-old rat. Note intense stain in the keratogenous zone and decreased reaction in the outer keratin layers. Barrnett and Seligman. ×100.

FIG. 5.—Combined SH and SS groups in upper jaw of 1-day-old rat. Note staining reaction similar to that in Fig. 1. Barrnett and Seligman. ×100.

FIG. 6.—Combined SH and SS groups in palate of 63-day-old rat. Note that staining reaction is similar to that in Fig. 3. Barrnett and Seligman. ×100.

FIG. 7.—Combined SH and SS groups in lower lingual gingiva of 54-day-old rat. The distribution is the same as that observed in the palate. Barrnett and Seligman. ×100.

and disulfide groups in the ground substance of the tunica propria than in the intercellular cement of the epithelium.

In the combined sulfhydryl and disulfide stain of Barrnett and Seligman the tunica propria of the 1-day-old animal was found to be weakly positive, but still lighter-staining than the epitheium. Some fibroblasts were weakly stained.

Total protein.—Protein, as shown by the brom-phenol blue technique (Mazia *et al.*) was normally found to be present in the epithelium in all tissues studied. The most intense staining was found to be present in the outer parakeratotic layer of the 1-day animal (Fig. 11) and in the stratum corneum of older animals (Fig. 12). Between 1 and 8 days of age there was an increase in the intensity of the reaction in the stratum Malpighii. There were no further changes which could be associated with aging (Fig. 12).

The intercellular material reacted strongly but no age changes were noticeable. Examination of the cell borders under oil immersion revealed that this staining reaction was due, at least in part, to the positive reaction of desmosomes.

Brom-phenol blue stained the tunica propria slightly more heavily in the older animal than in the younger, but comparatively more lightly than the staining reaction of the epithelium. Fibroblasts stained only slightly.

Desoxyribonucleic acid.—Desoxyribonucleic acid was present in similar amounts at all ages studied and was distributed throughout all layers of the epitheium, decreasing as the surface layers were approached. This was detected by the Feulgen reaction (Figs. 13, 14) and the Harris hematoxylin method of Jarrett. The presence of the acid was confirmed by its removal in control slides by incubation in desoxyribonuclease. In the 1-day-old rat, the nuclei in the basal cells and in the lower two or three layers of the spinosum stained strongly with the Feulgen reaction. Even the flattened, desiccated cells of the surface layer had strongly reactive, though flattened, nuclei. In all but the immature animals the outermost layers of the epithelium lack nuclei and appeared well keratinized. A decrease in the intensity of the staining of the nuclei from the basalis layer to the Malpighii was observed, followed by an increase in the intensity of the stain in the flattened corneal cells. Figures 15 and 16 are serial sections from the same tissue, with the latter showing the staining reaction for disulfides in the surface parakeratotic zone.

Ribonucleic acid.—Ribonucleic acid was found to be present predominantly in the basal-cell layer of the epithelium. The intensity of the staining reaction decreased as the corneum was approached. The distribution appeared to be the same throughout all the tissues studied, regardless of age or region (Figs. 17–20). The toluidine blue method of Brachet and the Harris hematoxylin method of Jarrett were employed in this study. Existence of the acid was determined by treatment of control slides with ribonuclease prior to staining. The results of the histochemical tests are summarized in Table 1.

DISCUSSION

The histochemical entities under consideration failed to reveal profound alterations which could be associated with the aging process. However, changes assumed to be developmental were present between 1- and 8-day-old animals. It is possible that transient cyclic or metabolic changes may exceed changes which might be attributed to aging. Another possibility is that the substances which were stained are of such impor-

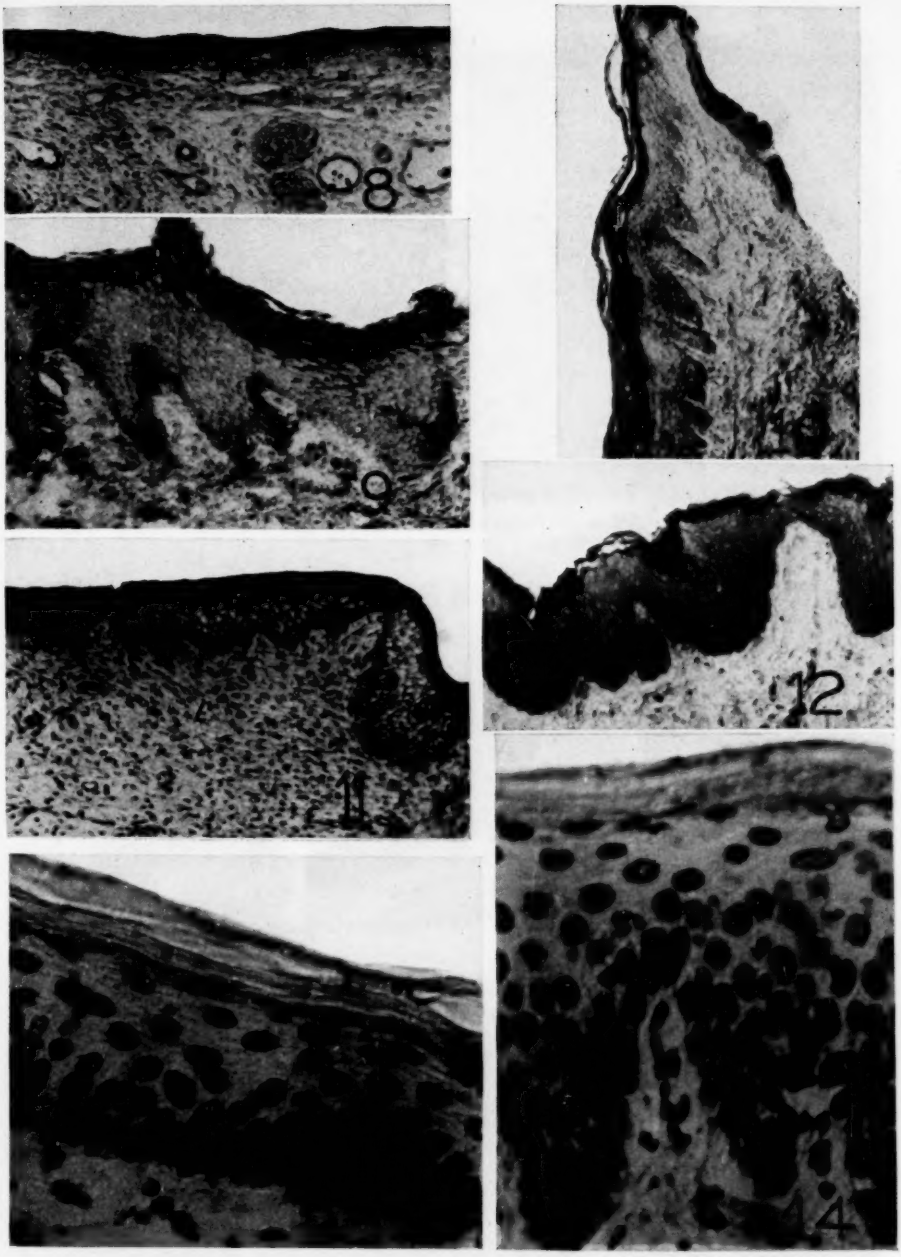


FIG. 8.—SS groups in lower jaw of a 1-day-old rat, utilizing blue tetrazolium after the method of Pearse. Note intense stain in corneum. ×100.

FIG. 9.—Lingual gingiva of 800-day-old rat, demonstrating SS groups in keratin and in basal cells of stratum Malpighii. Pearse. ×100.

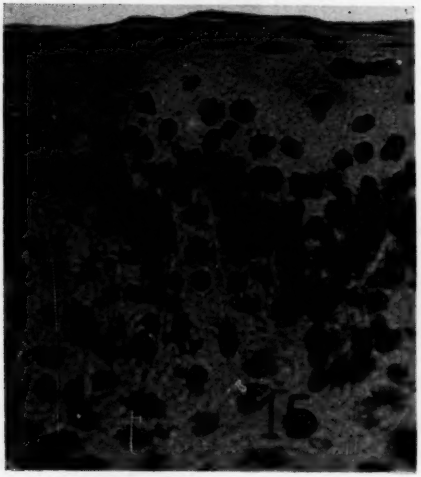
FIG. 10.—Palate gingiva of 800-day-old rat stained for SS after the method of Pearse. Note that distribution is the same as that found in the lower lingual gingiva. ×100.

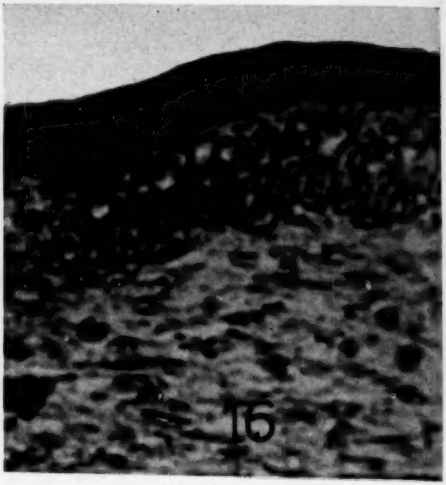
FIG. 11.—Localization of total protein in the upper jaw of 1-day-old rat. Note that the parakeratotic epithelium stains more heavily than the stratum Malpighii and the epithelium stains more heavily than the tunica propria. Mazia and Brewer stain. × 100.

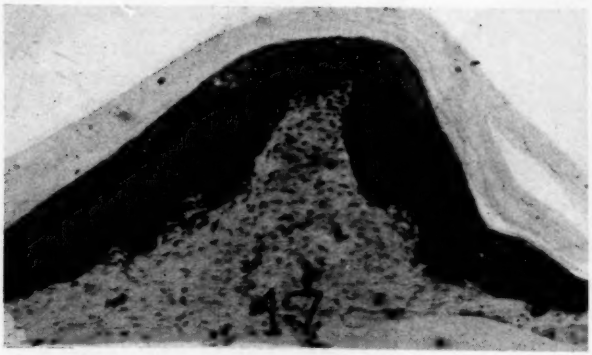
FIG. 12.—Total protein demonstrated in the palate of 850-day-old rat. Note that stratum corneum stains more heavily than the remainder of the epithelium. Mazia and Brewer stain. × 100.

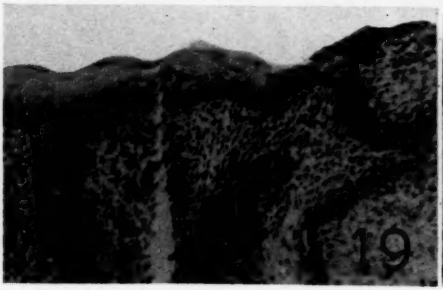
FIG. 13.—Feulgen reaction for DNA in palate cheek of 54-day-old hamster. Note that corneal cells are stained as intensely as the basal cells. ×300.

FIG. 14.—Same reaction in palate cheek of 540-day-old hamster. Note that the flattened corneal cells are as intensely stained as basal cells. Feulgen. ×300.









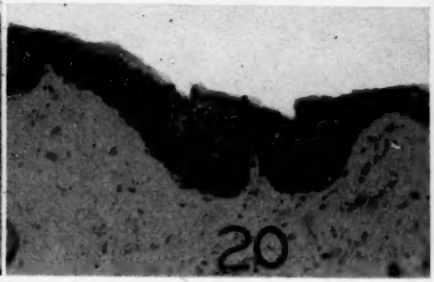


FIG. 15.—One-day-old rat upper jaw stained for DNA by the Feulgen technique. Note the flattened cells at periphery of epithelium indicative of parakeratosis. ×300.

FIG. 16.—One-day-old rat upper jaw stained for SS with blue tetrazolium by the method of Pearse. Note that a comparison with the previous figure demonstrates the presence of SS groups in the area of the parakeratotic zone. ×300.

FIG. 17.—Sagittal section of palate of 54-day-old hamster, showing decreased concentrations of RNA in the periphery of the epithelium. Toluidine blue. × 100.

FIG. 18.—Lingual gingiva of 450-day-old hamster, showing distribution of RNA. Toluidine blue. ×100.

FIG. 19.—Cross-section of 1-day-old rat upper jaw, showing distribution of RNA. Toluidine blue. ×100.

FIG. 20.—Palate cheek of 850-day-old rat, showing distribution of RNA. Toluidine blue. ×100.

tance to cellular vitality that no change can occur in life. A third possibility is that these techniques are not sufficiently refined to discern subtle alterations which may be associated with aging.

The outermost epithelial layer of the mucous membrane of the 1-day animals was strongly reactive for SH, SS, and protein groups. Examination of Feulgen sections revealed the presence of DNA in these cells. This would indicate that this area is not a true keratinized layer but rather parakeratotic. Examinations of sections of older animals were suggestive of a decrease in protein in the most peripheral layers of the

TABLE 1*

DISTRIBUTION OF HISTOCHEMICAL SUBSTANCES IN ORAL MUCOUS MEMBRANE OF RODENTS AND COMPARISON BETWEEN 1 AND 860 DAYS OF AGE

Substance	DNA	RNA	SH	SS	Protein	Age
Keratin	0	0	++++	++++	+++	860 days (absent in 1 day)
Prekeratin	++++	+	++	++++	++++	1 day and 860 days
Malpig. upper	+++	++	++	+	+	860 days (absent in 1 day)
Malpig. layer lower	+++	+++	++	+	+	1 day and 860 days
Basal layer	++++	+++	++	+	+	1 day and 860 days

* ++++ = strongly positive; +++ = moderately to strong; ++ = weak to moderate; + = weak; 0 = negative.

TABLE 2*

DIFFERENCES BETWEEN INTERCELLULAR CEMENT SUBSTANCE OF ORAL MUCOUS MEMBRANE

Method of Staining	Intercellular Substance of Epithelium	Ground Substance of Connective Tissue
Barrnett and Seligman for SH	+++	+
Barrnett and Seligman for SS	+++	+
Mazia and Brewer brom-phenol blue for total protein	+++	+

* Strongly positive, +++; moderately positive, ++; weakly positive, +; negative, 0.

corenum, although the SH and SS groups remained the same. Corresponding sections stained by the Feulgen method showed that there was no DNA present peripheral to the prekeratin layer, indicating that the superficial layers were completely keratinized. This would seem to indicate that the peripheral layer of the 1-day-old animal corresponded to the keratogenous zone of Giroud.[15]

According to the observations presented, there appears to be some indication of differences between the epithelial intercellular material and connective tissue ground substance (Table 2). Results of the brom-phenol blue, sufhydryl, and disulfide reaction of Barrnett and Seligman and disulfides as stained by the blue tetrazolium method revealed a strongly positive reaction in the area believed to be intercellular cement, with a weak reaction in the ground substance. The weak reaction of sulfhydryls in the tunica

propria is in agreement with Turesky and Glickman, in that they find a light reaction for sulfhydryls in the tunica propria and a stronger reaction in the fibroblasts.

These differences with the various stains suggest differences related to the keratinization process, for the positive staining reactions in the intercellular cement were similar to those found in the keratin and prekeratin layers. Apparently, the intercellular cement has more reactive proteins, which probably contain more cystine and cysteine than does the connective tissue ground substance.

Dewar reported no change in the DNA content of the gingiva associated with inflammation. Homogenate studies by Falzone and Shock[16] revealed no change in DNA content with age. The staining reaction for DNA in this investigation displayed small variations in intensity as the superficial layers were approached. The parakeratotic zone of the 1-day-old animal was intensely stained for DNA, being slightly heavier than the intensity of the stain exhibited in the basal layer. The keratinized layer in the adult was negative for DNA. Montagna *et al.*[17] reported that DNA decreased in intensity as the surface epithelium of skin was approached. In this investigation, a change in shape of the cells to a more flattened configuration was evident, but there was little difference in the intensity of the stain of the entire epithelium. The decrease in RNA in the cytoplasm in the cornified layers can be compared with the increase in protein, SH, and SS reactive groups in this region. It appears that the RNA synthesis precedes that of protein, for when there is high concentration of protein in the corneal layer, there is a diminution in the amount of RNA present. These results are in agreement with Korning,[18] who demonstrated this relationship of proteins and RNA in wing feathers.

SUMMARY AND CONCLUSIONS

The concentration of sulfhydryl, disulfide, and total protein does not appear to be altered by the aging process in the oral mucous membrane of rats and hamsters.

An area beneath the corneum stained intensely for sulfhydryls in the older animals. This area is apparently identical with the keratogenous zone of Giroud.

Desoxyribonucleic acid and ribonucleic acid in the oral mucous membranes of rodents does not vary with increasing age.

A decrease in the intensity of the Feulgen staining of the nuclei from the basali to the more superficial layer of the epithelium was observed, followed by an increase in the intensity of the stain in the flattened corneal cells.

The staining reaction for ribonucleic acid decreases in intensity from the basal layer of the epithelium to the stratum corneum.

Differences were detected between the intercellular cement substance of the epithelium and the connective tissue ground substance. A strong reaction was found in the intercellular substance for protein, sulfhydryls, and disulfides, in contrast to the weak reaction in the ground substance.

The parakeratotic zone of the 1-day-old animals stained identically with the keratinized area of the older animals for SH, SS, and protein groups. It is therefore suggested that an alteration in protein groups, other than SH and SS, are involved in the transformation of parakeratotic to completely keratinized tissue.

REFERENCES

1. Barrnett, R. J., and Seligman, A. M. Histochemical Demonstration of Sulfhydryl and Disulfide Groups of Protein, *J. Natl. Cancer Inst.*, **14**:769, 1954.
2. Turesky, S., Crowley, J., and Glickman, I. Histochemical Study of Protein Bound Sulfhydryl and Disulfide Groups of Normal and Inflamed Human Gingiva, *J. D. Res.*, **36**:255, 1957.
3. Dewar, N. R. Observations on the Composition and Metabolism of Normal and Inflamed Gingivae, *J. Periodont.*, **26**:29, 1955.
4. Turesky, S., Glickman, I., and Fisher, B. The Effect of Physiologic and Pathologic Processes upon Certain Histochemically Detectable Substances in the Gingiva, *J. Periodont.* **30**:116, 1959.
5. Castenfelt, F. *Toothbrushing and Massage in Periodontal Disease*. Stockholm: Nordisk Rotogravyer, 1952.
6. Lillie, R. D. *Histopathologic Technique: Practical Histochemistry*. New York: Blakiston Co., Inc., 1954.
7. Brachet, J. The Use of Basic Dyes and Ribonuclease for Cytochemical Detection of RNA, *Quart. J. Microscop. Sci.*, **94**:1, 1953.
8. Jarrett, A., and Hardy, J. A. The Value of Alcohol for Fixation of Skin, *Stain Technol.*, **32**:225, 1957.
9. Barrnett, R. J., and Seligman, A. M. Demonstration of Protein Bound Sulfhydryl and Disulfide Groups by Two New Histochemical Methods, *J. Natl. Cancer Inst.*, **13**:215, 1952 (abstr.).
10. ———. Histochemical Demonstration of Protein Bound Sulfhydryl Groups, *Science*, **116**:323, 1952.
11. Pearse, A. G. E. The Histochemical Demonstration of Cystine Containing Structures by Methods Involving Alkaline Hydrolysis, *J. Histochem. and Cytochem.*, **1**:460, 1953.
12. Mazia, D., Brewer, P. P., and Albert, M. The Cytochemical Staining and Measurement of Protein with Mercuric Bromphenol Blue, *Biol. Bull.*, **104**:57, 1953.
13. Barrnett, R. J. Histochemical Demonstration of Sites of Protein Bound Sulfhydryl and Disulfides, *Anat. Rec.*, **115**:280, 1953 (abstr.).
14. ———. The Histochemical Distribution of Protein Bound Sulfhydryl Groups, *J. Natl. Cancer Inst.*, **13**: Part 2, 905, 1952–53.
15. Giroud, A., and Leblond, C. P. The Keratinization of the Epidermis and Its Derivatives, Especially the Hair as Shown by X-Ray, *Ann. N.Y. Acad. Sci.*, **54**:613, 1951.
16. Falzone, J. P. F., Barrows, C. H., and Shock, N. W. Age and Polyploidy of Rat Liver Nuclei as Measured by Volume and DNA Content, *J. Gerontol.*, **14**:2, 1959.
17. Montagna, W., Eisen, A. E., Rademacher, A. H., and Chase, A. H. Histochemistry and Cytochemistry of Human Skin. VI. The Distribution of Sulfhydryl and Disulfide Groups, *J. Invest. Dermatol.*, **23**:22, 1954.
18. Korning, A. L. Histochemical Localization of Certain Constituents of the Developing Juvenile Wing Feather, *Am. J. Anat.*, **100**:17, 1957.

Studies on the Antibacterial Action of Human Saliva II. Observations on the Mode of Action of a Lactobacillus Bactericidin

BERNARD J. ZELDOW

Department of Periodontics and Endodontics, Division of Research, University of Washington, School of Dentistry, Seattle

In previous communications, Zeldow[1, 2] described a lactobacillus bactericidin found in pure parotid and submaxillary saliva. This factor required a dialyzable heat-stable co-factor for its activity. The non-dialyzable fraction was moderately stable over a wide range of pH, with maximum stability at pH 5.5. It appeared to be an endogenous product of the glands. Kerr and Wedderburn[3] also described an antibacterial system of saliva which appeared to be identical with this antilactobacillus system.

MATERIALS AND METHODS

Unless specified otherwise, the following materials and procedure were used.

Saliva.—Human parotid saliva was used in this investigation. Before application of the sterile collector,[4] 1 per cent tincture of iodine and 70 per cent alcohol were applied to the mucosa surrounding Stensen's duct. All specimens were stored at —30° C. until used.

Before use, the specimens were defrosted, passed through a Swinney filter, and the first 1-ml. aliquot discarded. The remaining sterile saliva was employed in the experiments, since it has been demonstrated to contain the same bactericidin titer as an unfiltered control saliva.[2]

Bactericidin assay method.—Lactobacillus acidophilus ATCC 4357 was used as the assay organism. The organism was maintained by subculture in Lactobacillus Selective Broth (LBS).* The technique employed for the quantitative measurement of salivary bactericidin has been given in a preceding publication.[2] For quantitative comparison, bactericidal activity is expressed in units. One unit is defined as the minimum amount of bactericidin present in 0.5 ml. of the dilution of saliva which will inhibit the growth of the bacteria contained in the 0.1 ml. of inoculum added.

Bactericidal tests.—The bactericidal effect of the lactobacillus bactericidin was was examined in the following media:

1. Medium I: LBS broth pH 5.5.

Received for publication June 20, 1960.

This investigation was supported by U.S.P.H.S. research grant D-771 from the National Institute of Dental Research, National Institutes of Health.

Presented in part before the 38th General Meeting of the International Association of Dental Research, Chicago, March 17–20, 1960.

* Available from Baltimore Biological Laboratories, Baltimore, Maryland.

2. Medium II: Medium I depleted by growing the organism in the broth at 37° C. for 24 hours and then separating them by centrifugation. This was repeated until the medium supported no further multiplication as indicated by turbidimetric and plating methods. After separation of the cells, the pH was readjusted to 5.5 and sterilized at 118° C. for 15 minutes.

3. Medium III: Medium II reconstituted by the addition of 20 gm. of glucose to each liter. The medium was then sterilized by filtration through a Seitz filter.

Log phase cells of the test organism were harvested from a 24-hour culture grown in Medium I and standardized in distilled water to contain approximately 3×10^7 organisms per milliliter. Two ml. of sterile saliva, 1.8 ml. of medium, and 0.2 ml. of cell suspension were immediately mixed and incubated in a 37° C. water bath. At various time intervals the number of viable cells was determined by the plate count method. Lactobacillus Selective agar was used for plating, and the number of colonies was counted after incubation at 37° C. for 48–96 hours. All counts were in duplicate. In all experiments, cultures of organisms, which had not been exposed to saliva but were otherwise treated in the same manner served as controls.

Adsorption technique.—A suspension of the test organism containing approximately 4×10^9 test cells per milliliter was prepared in Medium I. Five ml. of this suspension were incubated at 37° C. for 24 hours with 5 ml. of parotid saliva on a slowly rotating platform. Following this, the suspension was centrifuged and the supernate passed through a Swinney filter. The first milliliter of filtrate was discarded, and determination of the lactobacillus bactericidin was carried out on the remaining aliquot. A control was treated identically except that the organisms were not included in the broth which was incubated with the saliva.

Dialysis.—One volume of saliva was dialyzed against 15 volumes of deionized water at 4° C. for 24 hours.

Staining.—Both the Gram stain and the Chance cell-wall stain[5] were used to determine any alteration in staining capacity or morphology.

RESULTS

The effect of saliva on growing cells.—Typical results demonstrating the bactericidal effect of the saliva on *L. acidophilus* growing in Medium I are shown in Figure 1. In the presence of saliva, the organisms rapidly lost their viability after an initial period of multiplication amounting to one or two generations. These results suggested that the lactobacillus bactericidin is fully effective only against multiplying cells.

The effect of saliva on resting cells.—Temperature was used as a means of controlling the rate of multiplication of the organism in the presence and absence of saliva in Medium I. The results of these experiments are shown in Table 1. At 10° C. there was no discernible multiplication in the control culture, nor was there any noxious effect of saliva on the experimental cultures. At 27° and 47° C., multiplication occurred at a moderate rate, and the salivary factor likewise showed moderate antibacterial activity. At 37° C. multiplication was rapid, and the antibacterial action of the lactobacillus bactericidin was marked. As the rate of multiplication increased with temperature, the saliva exerted a proportionately increased killing effect. Thus, while 37° C. appeared to be the optimum growth temperature, it was also the temperature at which maximum killing occurred.

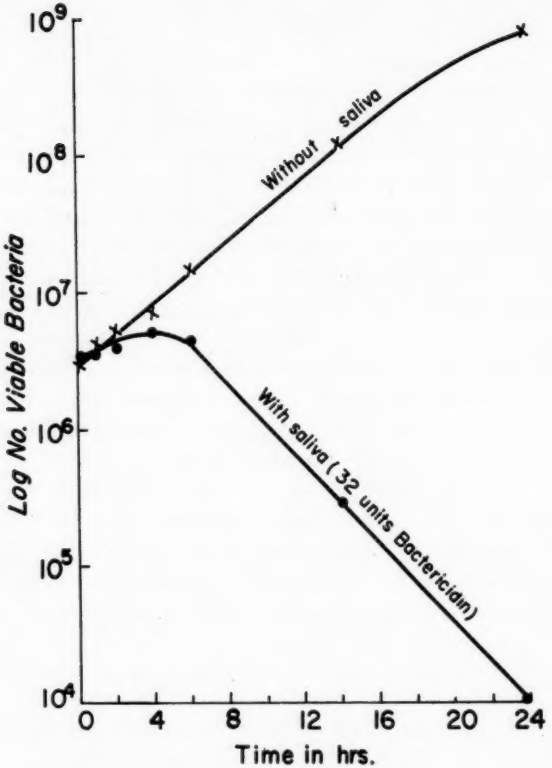


FIG. 1.—Viable cell counts of cultures of *L. acidophilus* ATCC 4357 with and without parotid saliva

TABLE 1

EFFECT OF TEMPERATURE ON THE VIABLE COUNT OF *Lactobacillus acidophilus* ATCC 4357 INCUBATED WITH AND WITHOUT PAROTID SALIVA

TEMP. (° C.)	24 HOURS		TEMP. (° C.)	24 HOURS	
	Fold Increase without Saliva	Fold Decrease with Saliva		Fold Increase without Saliva	Fold Decrease with Saliva
10.....	0	0	37.....	379	184
27.....	266	64	47.....	314	136

Another means of arresting the growth of the assay organism was necessary. A medium in which the assay organisms could be maintained in a resting state, i.e., without appreciable multiplication or death, was sought. Distilled water, saline, McIlvane's and Sorensen's buffers failed, as the organisms did not survive long in these solutions. However, Medium II served this purpose well.

The effect of the saliva on resting organisms in Medium II is shown in Figure 2. It is evident that in this medium the saliva containing the lactobacillus bactericidin did not exert any appreciable killing of the cells. However, in growth supporting Medium III, the saliva exerted a killing effect similar to that previously observed in Medium I.

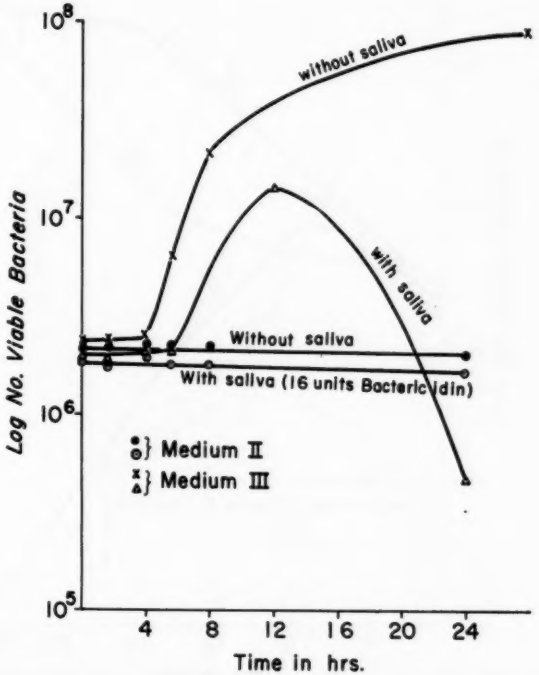


FIG. 2.—Viable cell counts of resting and growing *L. acidophilus* ATCC 4357 with and without saliva

The effect of bactericidin concentration.—The effect of various concentrations of the bactericidin on the assay organism is summarized in Figure 3. Curve *II* characterizes the growth of the organism in the presence of 1–32 units of salivary lactobacillus bactericidin. A temporary method of multiplication followed by rapid death again becomes apparent. In the range of 1–32 units of lactobacillus bactericidin per milliliter of saliva, the same pattern of lethal action on the organism was observed. There was no appreciable difference in the rate of killing within this range of concentration of the factor. Curve *III* demonstrates the activity of a one-half unit of lactobacillus bactericidin. After an initial period of rapid multiplication, the growth rate apparently became slowed, which was presumed to represent a period of bacteriostasis. This was

followed by the resumption of the initial rate of multiplication, which continued until the total number of organisms approximated that of the control at the end of 24 hours. Curve *IV* demonstrates the activity of one-quarter unit of the bactericidin. This curve simulated the control without saliva. The amount of bactericidin is apparently insufficient to exert any inimical effect on the growth of the susceptible organism.

The effect of dialyzing the saliva.—Dialyzed saliva has been shown by the quantitative assay method to lose its lactobacillus bactericidin activity.[2] Figure 4 dem-

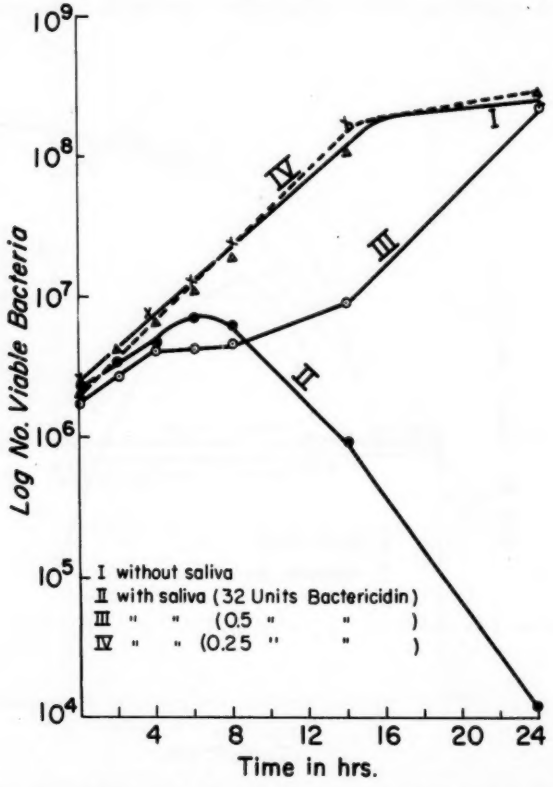


FIG. 3.—Viable cell counts of cultures of *L. acidophilus* ATCC 4357 with varying concentrations of parotid saliva.

onstrates the effect of the dialyzed saliva on the population of viable cells in the culture. With the exception of an initial slower rate of multiplication, the growth curve of organisms in the presence of dialyzed parotid saliva is similar to the control curve without saliva.

The effect of adsorption.—No diminution in the amount of lactobacillus bactericidin activity was noted in the saliva after incubation with viable sensitive cells. As compared with the control saliva, the saliva exposed to the assay organism for 24 hours demonstrated equal lactobacillus bactericidin activity per milliliter.

Staining and morphologic changes.—After exposure to the saliva, the organisms did

not appear to change morphologically, nor was the Gram reaction altered. Moreover, the Chance cell-wall stain did not reveal any changes.

DISCUSSION

Death of bacteria due to "unbalanced growth" is attributed to the limited impairment of certain biochemical processes. Other unrelated reactions function normally. Cohen and Barner[6] assigned the death of a thymine-requiring mutant of *E. coli* to unbalanced growth when placed in a complete medium except for the omission of

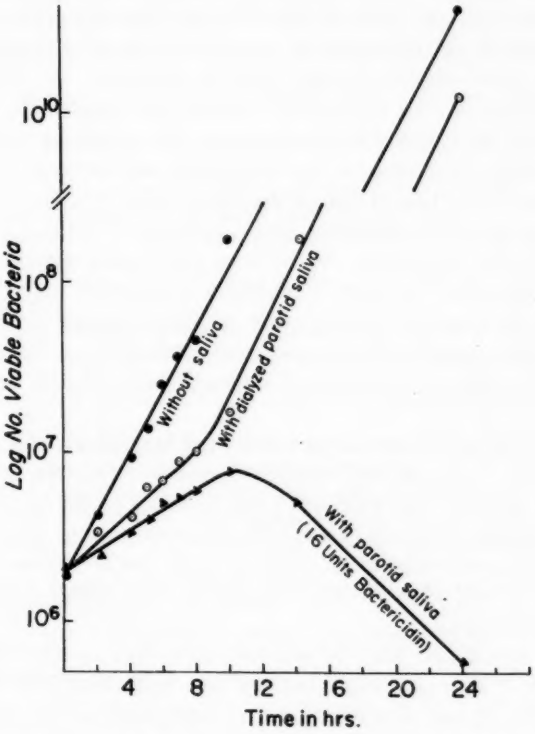


FIG. 4.—Comparison of the effect of dialyzed and non-dialyzed parotid saliva on the cell count of cultures of *L. acidophilus* ATCC 4357.

thymine. Following a temporary increase in the number of viable cells, death of the organisms occurred during a period in which the cells were observed to synthesize all their constituents with the exception of deoxyribonucleic acid. Other examples of unbalanced growth appear in the literature.[7, 8]

In the present investigations, death of the sensitive organism in the presence of lactobacillus bactericidin also followed an initial temporary period of multiplication. Such a sequence was highly suggestive of unbalanced growth in which an essential biochemical reaction is impaired. In accord with previous findings, it was to be expected that if the lethal action of the lactobacillus bactericidin is truly due to unbalanced growth, the agent should not exert an inimical effect on resting cells. Such

were the findings demonstrated in systems in which growth was suppressed either by unfavorable temperature or by the use of depleted medium. However, in the former experiment in which it was shown that maximum killing occurred at the optimum growth temperature, another interpretation can be justly made. It is possible that if the antibacterial agent is an enzyme, the temperature of its maximum activity may be nearly identical with the optimum temperature for the growth of the culture. It was necessary, therefore, to test the effect of the saliva on organisms held in a resting state by some other method. Depleted media served this purpose well, wherein it was shown that the salivary factor exerted no apparent killing effect on the organisms. Moreover, when this was controlled by reconstituting the medium by the addition of glucose, the growth of the organisms and the action of the antibacterial agent were similar to the results observed in the complete medium.

The results indicate that the salivary lactobacillus bactericidin exerts a lethal effect on sensitive cells only when they are growing. The action of this salivary component upon non-dividing organisms, on the other hand, was without obvious effect. In this respect, the mode of action of this antibacterial factor is similar to other antibacterial agents, such as penicillin, spermine, and polylysine.[9–11] Although death due to lactobacillus bactericidin apparently results from unbalanced growth, no evidence is presented suggesting what the basic biochemical impairment may be.

Salivary antilactobacillus systems with different physical properties have been reported in the literature.[12, 13] The presence of more than one lactobacillus bactericidin in the saliva is a distinct possibility. Continued studies should be done to clarify this problem.

It has been shown that a minimum amount of lactobacillus bactericidin is necessary for lethal action. Under natural conditions, saliva bathes the oral mucosa as a thin film, and it is possible that sufficient concentrations of the factor are not present to have a detrimental effect on susceptible micro-organisms. However, Williams and Powlen,[14] using a roller-tube technique to simulate the in vivo condition, reported that *Lactobacillus,* as well as other micro-organisms, was unable to survive and multiply in sterile parotid saliva.

It has been established that the oral microbial flora is relatively characteristic for an individual.[15, 16] It is postulated that salivary antibacterial systems such as the one described in the present investigation may contribute to the regulation of the oral flora and play an important part in the natural defense mechanisms of the oral cavity. Since this agent probably represents only one of a large number of factors contributing to the antimicrobial defense system of the oral cavity, allied mechanisms must not be overlooked.

SUMMARY

Some basic observations on the mode of action of a salivary lactobacillus bactericidin were made.

The lethal effect of this agent on actively multiplying *L. acidophilus* ATCC 4357 is apparently due to unbalanced growth. This was supported by the observations that killing of the organism was not accomplished by the saliva until an initial period of multiplication had occurred and that the antibacterial factor was without effect on resting organisms.

The minimal bactericidal concentration of the lactobacillus bactericidin was 1 unit per milliliter. One-half unit per milliliter showed temporary stasis, whereas one-quarter unit per milliliter showed no inhibitory effect.

The bactericidin apparently does not alter the morphology or Gram reaction of the organism. It is not adsorbed by the organism.

The author wishes to thank Dr. Howard C. Douglas and Dr. Russell S. Weiser for their many helpful suggestions in the preparation of this text. The technical assistance of Mr. Nelson Hill is gratefully acknowledged.

REFERENCES

1. Zeldow, B. J. Antibacterial Activity of Mixed and Parotid Human Saliva. A Preliminary Report, *J. D. Res.*, **34**:737, 1955.
2. ———. Studies on the Antibacterial Action of Human Saliva. I. A Bactericidin for Lactobacilli, *ibid.*, **38**:798, 1959.
3. Kerr, A. C., and Wedderburn, D. L. Antibacterial Factors in the Secretions of Human Parotid and Submaxillary Glands, *Brit. D. J.*, **105**:321, 1958.
4. Curby, W. A. Device for Collection of Human Parotid Saliva, *J. Lab. Clin. Med.*, **41**:493, 1953.
5. Chance, H. L. A Bacterial Cell Wall Stain, *Stain Technol.*, **28**:205, 1953.
6. Cohen, S. S., and Barner, H. D. Studies on Unbalanced Growth in *Escherichia coli*, *Proc. Natl. Acad. Sci.*, **40**:885, 1954.
7. Ridgeway, G. J., and Douglas, H. C. Unbalanced Growth of Yeast Due to Inositol Deficiency, *J. Bacteriol.*, **76**:163, 1958.
8. Lederberg, J. Bacterial Protoplasts Induced by Penicillin, *Proc. Natl. Acad. Sci.*, **42**:574, 1956.
9. Hobby, G. E., Meyer, K., and Chaffee, E. Observation on the Mechanism of Action of Penicillin, *Proc. Soc. Exptl. Biol. Med.*, **50**:281, 1942.
10. Razin, S., and Rozansky, R. Mechanism on the Antibacterial Action of Spermine, *Arch. Biochem.*, **81**:36, 1959.
11. Katchalski, E., Bichowski-Slomnitzki, L., and Volcani, B. E. The Action of Some Water-soluble Poly-α-amino Acids on Bacteria, *Biochem. J.*, **55**:671, 1953.
12. Hill, T. J. A Salivary Factor Which Influences the Growth of *L. acidophilus* and Is an Expression of Susceptibility or Resistance to Dental Caries, *J.A.D.A.*, **26**:239, 1939.
13. Green, G. E. A Bacteriolytic Agent in Salivary Globulin of Caries-Immune Human Beings, *J. D. Res.*, **38**:262, 1959.
14. Williams, N. B., and Powlen, D. O. Human Parotid Saliva as a Sole Source of Nutrient for Microorganisms, *Arch. Oral Biol.*, **1**:48, 1959.
15. Kraus, F. N., and Gaston, C. Individual Constancy of Numbers among the Oral Flora, *J. Bacteriol.*, **71**:703, 1956.
16. Hurst, V. Bacterial Flora of the Mouth, *J. Periodont.*, **27**:87, 1956.

Studies on the Amino Acid Decarboxylases of Oral Lactobacilli

WILLIAM R. BIBB and W. R. STRAUGHN

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill

The first complete study of the occurrence and distribution of the bacterial amino acid decarboxylases was carried out by Gale[1, 2] and Gale and Epps,[3] primarily with strains of *Escherichia coli and Clostridium perfringens*. However, strains of the genus *Lactobacillus* were not included. Lagerborg and Clapper[4] published the first study of amino acid decarboxylases in oral lactobacilli. Of the 33 strains of oral lactobacilli tested, 12 had the ability to produce carbon dioxide from arginine, 3 from glutamic acid, 3 from tyrosine, 1 from histidine, and 1 from ornithine. Tests of the reaction products of one of the strains producing carbon dioxide from arginine indicated the presence of ammonia. For this reason they believed that the enzyme acting on arginine was a "dihydrolase." They also felt that all the strains of lactobacilli tested were devoid of true arginine decarboxylase.

Arginine "dihydrolase" comprises two enzyme systems which catalyze the metabolism of arginine with the liberation of 2 moles of ammonia and 1 mole each of carbon dioxide and ornithine per mole of arginine.[5, 6] The name "arginine desimidase" was proposed for the enzyme catalyzing the breakdown of arginine to citrulline and ammonia.[5] "Citrulline ureidase" was proposed for the enzyme system catalyzing the breakdown of citrulline to ammonia, carbon dioxide, and ornithine.[7]

The present study was designed to investigate the distribution of amino acid decarboxylases among 58 recently isolated strains of oral lactobacilli and to determine the optimum conditions for the formation of these enzymes. Some of the factors affecting their activity were investigated. An attempt was also made to distinguish desimidase and citrulline ureidase activity from the amino acid decarboxylase activity of these lactobacilli.

MATERIALS AND METHODS

The organisms used for these studies were obtained from paraffin-stimulated salivas, root canals, and mouth washings from patients at the University of North Carolina School of Dentistry. These samples were streaked on LBS* agar and incubated for 4 days at 37° C. LBS agar[8] is a highly selective medium and supports the growth of few organisms other than lactobacilli. Approximately 100 isolates were replated until pure cultures were established. Those organisms which were Gram-positive, catalase-negative bacilli were retained as lactobacillus species. The isolates were main-

This study was supported by Research Grant D-476 from the N.I.H., U.S. Public Health Service.
Received for publication July 5, 1960.

* Baltimore Biological Laboratory, Inc.

tained as stock cultures in Micro Assay Culture Agar* at 2°–6° C. following 24–48 hours' incubation at 37° C. All strains were also lyophilized. These isolates were grown in Difco Micro-inoculum broth at 30° and 37° C., and titratable acidity was determined at the end of 24 and 72 hours with 0.1 N sodium hydroxide to a phenolphthalein end-point. The gas production was determined in Micro-inoculum agar containing 2 per cent glucose. Splitting of the agar stab by the organisms was recorded as gas production.

For testing enzymatic activity of the organisms, a cell suspension was prepared by centrifuging the medium II culture at 3° C. and washing the cells twice with cold distilled water. After washing, the cells were suspended in enough cold distilled water to give a cell suspension of 16 mg. dry weight per ml. as determined by a standard turbidity–dry-weight curve.

The histidine, lysine, and glutamic acid decarboxylase activities and the carbon dioxide production from arginine of these lactobacilli were determined manometrically by the standard Warburg technique. Air was used as the gas phase, since no difference was observed when the gas phase was nitrogen. Experiments were carried out at 37° C. The histidine and glutamic acid decarboxylases were determined at pH 4.5, and the lysine decarboxylase and carbon dioxide production from arginine at pH 5.3. The main cup contained 1.5 ml. of 0.05 M phthalate buffer and 1 ml. of the cell suspension to be tested while the side arm contained 0.5 ml. of a 3×10^{-2} M concentration of amino acid substrate in phthalate buffer. Expressed as microliters of carbon dioxide liberated from the substrate per hour per milligram dry weight of organisms, Q_{CO_2} values were calculated from the initial maximum rates of carbon dioxide production.

Qualitative determination of the amino acids† was accomplished by paper chromatography, using a descending paper-strip system with 77 per cent aqueous ethyl alcohol saturated with diethylamine as solvent. The amino acid spots were developed with 0.3 per cent ninhydrin in acetone. Citrulline was further identified with Ehrlich's reagent, and ornithine with vanillin.[9] Ammonia was determined with Nessler's reagent.

RESULTS

The initial study in this investigation was the determination of a medium that would be suitable for growth and decarboxylase activity of lactobacilli.[10, 11] *Lactobacillus* strain 41 was found to produce considerable gas in 24–48 hours at 37° C., and, as it was felt that a heterofermentative organism would be more likely to produce amino acid decarboxylases, this organism was employed in this initial study. The compositions of the various growth media and the growth responses of strain 41 are recorded in Table 1. The media were all adjusted with sodium hydroxide to a final pH of 6.5 except number V, which was used at a pH of 5.8. One hundred ml. amounts of each medium dispensed in 250-ml. flasks were inoculated and incubated for 48 hours at 37° C.

Medium II was chosen for subsequent use because it was relatively simple and allowed maximum acid production, a factor which Gale[12] has indicated as important

* Difco Laboratories, Inc., Detroit, Michigan.

† The amino acids and pyridoxal hydrochloride were obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

in the formation of amino acid decarboxylases. Strain 41 grown in medium II yielded maximum growth and acid production after 48 hours' incubation at 37° C. This did not hold true for all the strains tested, the time of incubation necessary to achieve maximum growth and acid production varying with different strains from 24 to 96 hours.

TABLE 1

GROWTH AND ACID PRODUCTION OF LACTOBACILLUS STRAIN 41 IN VARIOUS MEDIA

Medium	Growth (Mg Dry Wt Cells/Ml)	Final pH
I. 1% Bacto-casitone 2% Glucose	0.9	4.4
II. 1% Casein hydrolyzate powdered—acid-hydrolyzed, salt- and vitamin-free (Nutritional Biochemicals Co.) 2% Glucose 0.1% Bacto-yeast extract	4.8	3.75
III. 1% Casein hydrolyzate powdered—acid-hydrolyzed, salt- and vitamin-free (Nutritional Biochemicals Co.) 2% Glucose 0.5% Bacto-yeast extract	6.9	3.8
IV. 1% Bacto-casamino acids 2% Glucose	0.025	5.8
V. 0.5% Bacto-proteose peptone No. 3 2% Bacto-yeast extract 1% Glucose 0.2% Potassium dihydrogen phosphate 0.01% Tween 80* polyoxyethylene sorbitan monooleate	5.6	4.2
VI. 0.5% Bacto-proteose peptone No. 3 2.0% Bacto-yeast extract 1.0% Glucose 0.2% Potassium dihydrogen phosphate 0.01% Tween 80	5.3	4.2
VII. 3.7% Bacto-micro inoculum broth	5.35	4.1
VIII. 0.5% Bacto-proteose peptone No. 3 2.0% Bacto-yeast extract 1.0% Glucose 0.01% Tween 80	5.35	3.8
IX. 0.5% Bacto-proteose peptone No. 3 2.0% Bacto-yeast extract 2.0% Glucose 0.2% Potassium dihydrogen phosphate 0.01% Tween 80	5.3	4.2

* Atlas Powder Company, Wilmington, Delaware.

Lactobacillus strain 3 (LB-3) was found to produce carbon dioxide only from histidine and arginine of the amino acid substrates tested and was chosen for further study to determine the optimum conditions for formation of these enzymes. In an effort to increase the carbon dioxide production from arginine by this organism, 0.1 per cent *l*-arginine was added to medium II prior to autoclaving. The cells grown in this arginine-enriched medium had a Q_{CO_2} of 5.0, while those grown in medium II alone had a Q_{CO_2} of 1.5 (Table 2).

The data revealed in Table 2 show that pyridoxal hydrochloride had a slight inhibitory effect on carbon dioxide production from arginine. Furthermore, cells grown in medium II plus 0.1 per cent *l*-arginine exhibited greater activity when tested at 37° C. than at 30° C. Pyridoxal hydrochloride, when similarly tested, was found to have no

effect on histidine decarboxylase activity. Results with pyridoxal phosphate were the same as those obtained with pyridoxal hydrochloride. The addition of *l*-tryptophan (100 mg. per liter) to medium II increased the total cell crop but had no effect on histidine decarboxylase activity. It was subsequently found that the additional stimulus to growth provided by *l*-tryptophan addition to medium II could be equaled by raising the yeast-extract concentration from 0.1 to 0.5 per cent.

The effect of growth temperature on carbon dioxide production by LB-3 grown in medium II at 26°, 30°, and 40° C. was tested, with arginine, histidine, ornithine,

TABLE 2

CARBON DIOXIDE PRODUCTION FROM ARGININE BY STRAIN LB-3 IN THE PRESENCE AND ABSENCE OF PYRIDOXAL HYDROCHLORIDE

GROWTH MEDIUM	PYRIDOXAL HCl FINAL CONCENTRATION 7.8×10^{-4} M	Q_{CO_2}	
		30° C.	37° C.
Medium II	Absent	1.5	1.5
	Present	1.1	1.0
Medium II+0.1% *l*-arginine	Absent	3.6	5.0
	Present	3.0	5.0

TABLE 3

EFFECT OF ADDED AMINO ACIDS IN GROWTH MEDIUM ON CARBON DIOXIDE PRODUCTION BY STRAIN LB-3

ADDITIONS TO MEDIUM III	Q_{CO_2}				
	Arginine	Histidine	Lysine	Glutamic Acid	Ornithine
None	1.3	41.5	1	1	0
0.1% *l*-Arginine	7.75	.....	.....	.....	.....
0.1% *l*-Histidine	.....	39.2	.....	1	.....
0.1% *l*-Lysine	.....	.....	1	.....	.....
0.1% *l*-Glutamic acid	.....	32.0	.....	1	.....
0.1% *l*-Ornithine	.....	.....	.....	.....	0

and glutamic acid as substrates. Best activities were found in those cells grown at 37° C.

Since the addition of 0.1 per cent *l*-arginine to medium II increased the carbon dioxide production from arginine, the stimulatory effect of *l*-glutamic acid, *l*-histidine, *l*-lysine, *l*-ornithine, and *l*-arginine added individually to the growth medium on the carbon dioxide produced by the corresponding amino acids was tested. Each of the five amino acids was added separately to liter flasks containing 600 ml. of medium III and cultured at 37° C. for 48 hours. The results are shown in Table 3.

Organism LB-3 had no ornithine decarboxylase activity when grown in medium III alone or with added *l*-ornithine. It was decided that the medium suggested by Rodwell[11] for ornithine decarboxylase should be tried before assuming LB-3 to be devoid of

this enzyme. The Rodwell medium is essentially medium III with the addition of pyridoxamine, folic acid, and thymine. The cells grown in this medium plus 0.1 per cent *l*-ornithine were tested at 37° C. and pH 5.3 in the presence and absence of pyridoxal hydrochloride. No ornithine decarboxylase activity was detected in either system, although an aliquot of the cells tested for carbon dioxide production from arginine showed a Q_{CO_2} value of 3.0.

TABLE 4

EFFECT OF SURFACE AREA OF MEDIUM AND AERATION ON CARBON DIOXIDE PRODUCTION FROM ARGININE AND HISTIDINE BY STRAIN LB-3

GROWTH CONDITIONS	SURFACE AREA (CM.²)	GROWTH (MG DRY WT CELL /ML)	pH	Q_{CO_2}	
				Histidine	Arginine
300 ml. broth in 500-ml. flask...	63.62	1.3	4.0	43.5	3.2
200 ml. broth in 500-ml. flask...	83.32	1.1	4.0	43.0	3.2
500 ml. broth in 2,800-ml. Fernback flask.....	323.59	1.9	4.0	29.0	3.5
500 ml. broth with air bubbled through sintered glass disk continuously.....	.....	2.5	3.8	17.2	5.0

TABLE 5*

CARBON DIOXIDE PRODUCTION FROM HISTIDINE, GLUTAMIC ACID, LYSINE, AND ARGININE BY 58 STRAINS OF ORAL LACTOBACILLI

SOURCE OF ORGANISMS	HISTIDINE			GLUTAMIC ACID			LYSINE			ARGININE		
	+	±	−	+	±	−	+	±	−	+	±	−
Saliva..... (Paraffin stimulation)	1	11	15	3	15	9	1	8	18	12	9	6
Mouth wash.....	1	1	18	0	2	18	0	0	20	12	2	6
Root canal.....	1	1	9	1	2	8	0	0	11	3	1	7
Total.....	3	13	42	4	19	35	1	8	49	27	12	19

* $+ = Q_{CO_2} > 2.5$; $\pm = Q_{CO_2} < 2.5 > 1$; $-$ = no measurable activity.

To determine the effect of oxygen tension during growth on enzyme formation, four flasks of medium III were inoculated with LB-3 and incubated at 37° C. for 48 hours. The results are shown in Table 4. Aeration decreased the carbon dioxide production from arginine from a Q_{CO_2} value of 43.5 to 17.2.

Using the optimum conditions for the production of amino acid decarboxylases, incubation at 37° C. in medium III to the point of maximum acid production, 58 strains of oral lactobacilli were tested for amino acid decarboxylases and carbon dioxide production from arginine. The four amino acids tested were *l*-histidine, *l*-arginine, *l*-lysine, and *l*-glutamic acid. These amino acids have been found to be those most commonly attacked by oral lactobacilli.[4] The results are recorded in Table 5.

During the course of this investigation it was found that certain strains of oral lactobacilli produced ammonia in addition to carbon dioxide from arginine. With urea as the substrate, no carbon dioxide was released by LB-3; therefore, this organism did not contain urease.

On the assumption that the arginine dihydrolase enzyme system might be the source of ammonia and carbon dioxide released from arginine by LB-3, the end products were identified chromatographically. With arginine as the substrate, arginine, ornithine, and citrulline were separated from the reaction mixture, and, with citrulline as the substrate, citrulline and ornithine were separated from the reaction mixture.

DISCUSSION

An attempt at defining the optimum conditions for amino acid decarboxylase production in oral lactobacilli led to several findings.

A uniform period of incubation was found to be an unsatisfactory method for obtaining optimum enzyme production in the species studied and was replaced by the determination of maximum acid production by a growing culture as an index of enzyme production. This correlates with the work of Gale and Epps,[3] who found that bacteria, grown in an acid medium, attacked amino acids by decarboxylation.

The presence of the specific substrate increased the carbon dioxide production from arginine but had no effect on histidine, glutamic acid, or lysine decarboxylase activities. The addition of histidine to that already present in the casein hydrolyzate of the growth medium had an inhibitory effect on carbon dioxide production.

Oral lactobacilli, on primary isolation, are usually found to be micro-aerophilic. Possibly because of the continued cultivation under aerobic conditions, the strain LB-3 grew quite well under conditions of increased aeration. However, the activity of histidine decarboxylase decreased as the aeration increased. The carbon dioxide production from arginine was not appreciably affected by increased aeration. The difference in the effect of aeration on the production of histidine decarboxylase and carbon dioxide production from arginine may be accounted for by the fact that arginine decarboxylase is not involved.

When the 58 strains of oral lactobacilli employed in this investigation were tested manometrically, carbon dioxide was produced most frequently from arginine. Glutamic acid decarboxylase was next in frequency of occurrence, followed by histidine and, finally, lysine decarboxylase. There was no pattern of occurrence of the enzymes in groups, however. Only 3 strains had a Q_{CO_2} greater than 2.5 with histidine as the substrate. The proposal of a histamine relationship with periodontitis is difficult to evaluate on the basis of the present findings with oral lactobacilli.

It is of interest that 67 per cent of the organisms tested produced carbon dioxide from arginine. It was found that every organism which produced carbon dioxide from arginine also liberated ammonia. Arginase was ruled out in LB-3 by testing the organism with urea as the substrate. In no instance was ammonia or carbon dioxide produced —products one would expect if urease were present. In the presence of arginine as the substrate, citrulline, ornithine, ammonia, and carbon dioxide were the end products of the reaction. With citrulline as the substrate, ornithine, ammonia, and carbon dioxide were the end products. The enzyme system splitting arginine to citrulline, ornithine,

ammonia, and carbon dioxide in the LB-3 strain was identified as arginine desimidase and citrullinase.

On the basis of evidence presented in this paper and similar findings by Lagerborg and Clapper,[4] it would seem that oral lactobacilli might not produce a true arginine decarboxylase.

SUMMARY

The optimum conditions for the production of amino acid decarboxylases by oral lactobacilli were determined. They included incubation at 37° C., harvesting when the point of maximum acid production was reached, and growth in a medium composed of 0.5 per cent Bacto-yeast extract, 2 per cent glucose, and 1 per cent powdered casein (acid-hydrolyzed, vitamin- and salt-free). Using these conditions, 58 strains of oral lactobacilli were tested manometrically for the presence of four amino acid decarboxylases. Glutamic acid decarboxylase was the most frequently occurring decarboxylase, followed by histidine and, finally, lysine decarboxylases. Sixty-seven per cent of the organisms produced carbon dioxide from arginine, and it was concluded that arginine decarboxylase was not involved but that the carbon dioxide production resulted from the action of arginine desimidase and citrullinase.

REFERENCES

1. Gale, E. F. The Production of Amines by Bacteria. I. The Decarboxylation of Amines by Strains of *E. coli*, *J. Biol. Chem.*, **34**:392, 1940.
2. ———. Enzymes Concerned in the Primary Utilization of Amino Acids by Bacteria, *Bacteriol. Revs.*, **4**:135, 1940.
3. Gale, E. F., and Epps, H. M. R. The Effect of the pH of the Medium during Growth on the Enzymatic Activities of Bacteria and the Biological Significance of the Change Produced, *J. Biol. Chem.*, **36**:600, 1942.
4. Lagerborg, B. Z., and Clapper, W. E. Amino Acid Decarboxylases of Lactic Acid Bacteria, *J. Bacteriol.*, **63**:393, 1952.
5. Roche, J., and Lacombe, G. Sur l'argininedesimidase et sur la formation enzymatique de citrulline par les levures, *Biochim. Biophys. Acta*, **9**:687, 1952.
6. Schmidt, G. C., Logan, M. A., and Tytell, A. A. The Degradation of Arginine by *Clostridium perfringens*, *J. Biol. Chem.*, **198**:771, 1952.
7. Slade, H. D. Hydrolysis of Arginine by Soluble Enzymes of *Streptococcus faecalis*, *Arch. Biochem. Biophys.*, **42**:204, 1953.
8. Rogosa, M., Mitchell, J. A., and Wiseman, R. F. A Selective Medium for the Isolation and Enumeration of Oral and Fecal Lactobacilli, *J. Bacteriol.*, **62**:132, 1951.
9. Block, R. J., Durrum, E. L., and Zweig, G. *A Manual of Paper Chromatography and Paper Electrophoresis*, pp. 92, 97, 112. New York: Academic Press, 1955.
10. Rodwell, A. W. The Histidine Decarboxylase of a Species of *Lactobacillus:* Apparent Dispensability of Pyridoxine Phosphate as a Co-Enzyme, *J. Gen. Microbiol.*, **8**:233, 1953.
11. ———. The Factors Affecting the Activation of the Ornithine Apo-Decarboxylase of a Strain of *Lactobacillus*, *ibid.*, p. 238, 1953.
12. Gale, E. F. The Bacterial Amino Acid Decarboxylases, *Advances in Enzymology*, **6**:1, 1946.

The Comparative Distribution of Glycogen, Succinic Dehydrogenase, and Acid Mucopolysaccharides in Different-aged Animals

JULES KLINGSBERG, LOUIS A. CANCELLARO,† and EARL O. BUTCHER*

Departments of Anatomy, College of Dentistry and the Graduate School of Arts and Science, New York University, and the Hahnemann Medical College and Hospital of Philadelphia

The presence of glycogen, succinic dehydrogenase,[1, 2] and acid mucopolysaccharides in the oral mucous membrane has been reported in several recent investigations. Previous studies have described a direct relationship,[3] an inverse relationship,[4, 5] and a lack of relationship[6, 7] between keratinization and glycogen. The distribution of mucopolysaccharides in the epithelial intercellular cement and connective tissue ground substance of the oral mucous membrane has likewise been determined by several investigators.[8–10]

This histochemical investigation was undertaken to compare the distribution of these substances and to relate them to aging and, where possible, to keratinization in the oral mucous membranes of rats and hamsters. Furthermore, the oral tissues of two 3-year-old monkeys were studied to determine whether a similar distribution of glycogen and succinic dehydrogenase existed in higher mammals.

MATERIALS AND METHODS

One hundred male Long-Evans rats, 75 male Syrian hamsters, and 2 female rhesus monkeys (*Macaca mulatta*) were used in this investigation. Tissues were taken from rats ranging in age from 1 to 900 days and from hamsters 1–540 days, since the latter seldom survived to an older age. The 1-day-old group consisted of 30 rats and 25 hamsters; the 8-day, of 30 rats and 25 hamsters; the 42–63-day, of 30 rats and 25 hamsters; and the oldest group, of 5 540-day-old hamsters and 10 850–900-day-old rats. The 2 monkeys were 2–3 years old. The rats and hamsters were sacrificed by cranial trauma, while biopsy specimens were taken from the monkeys under nembutal anesthesia. All tissues were fixed in alcoholic formalin (AAF) (85 parts absolute alcohol, 5 parts glacial acetic acid, and 10 parts 40 per cent formaldehyde) for 4 hours at 4° C. Paraffin with a melting point of 56°–58° C. was used for imbedding. Rec-

Part of this investigation was presented to the Graduate School of Arts and Science, New York University, in partial fulfilment for the requirements for the degree of Doctor of Philosophy in the Department of Anatomy.

This study was supported by U.S.P.H.S. research grant D-366 from the National Institute of Dental Research, National Institutes of Health.

Received for publication July 5, 1960.

*Postdoctoral Research Fellow of the National Institute of Dental Research.

† Presently at the Hahnemann Medical College and Hospital, Philadelphia.

tangular strips of gingiva were dissected to include both the marginal and the vestibular gingiva. Three specimens were taken from the palate—one from the mid-line and two bilaterally from the gingival portion of the palate. Biopsies were also taken from the cheek pouch of the hamsters and from gingiva and palate of the monkeys. All tissues were sectioned at a thickness of 8 μ. Twenty-five serial sections were made from each specimen, and representative sections of each tissue were stained in hematoxylin and eosin. Keratinization was evaluated according to the classification of Castenfelt,[11] whose criterion is based on nuclear morphology and staining reaction.

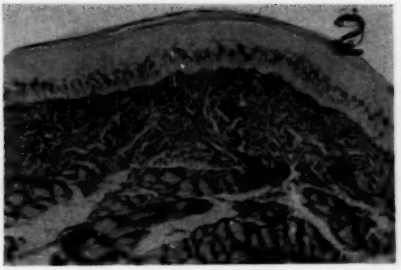
The following histochemical techniques were utilized in this investigation. The Hotchkiss aqueous periodic acid–Schiff reagent was used for basement membrane and glycogen determinations.[12] Care was taken to avoid dissolving the mucopolysaccharides present in the tissues by maintaining the temperature of the hot plate at 37° C. while spreading the tissue and by the subsequent removal of the slides from the hot plate, thereby permitting the tissues to air-dry for 24 hours. To determine the presence of glycogen, control tissue sections were digested in a 0.1 per cent solution of malt diastase buffered with phosphates to pH 6.0 overnight at 37° C. Since malt diastase is not entirely specific for glycogen because of small traces of ribonucleases present, dilute solutions, in the order of 1/1,000, were utilized. Since the PAS method showed no glycogen in the monkey, the Best carmine method was used to verify this absence.

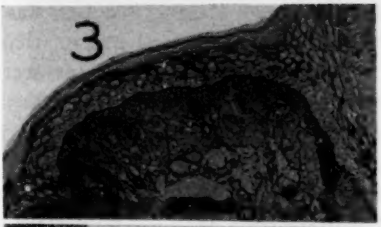
Succinic dehydrogenase was determined by using the techniques of Padykula and of Formisano and Montagna, who employed the tetrazolium salts. In this action the sodium succinate, which is the substrate, is dehydrogenated.[13, 14] The tetrazolium salts, normally colorless, are thereby reduced to a colored salt, which indicates the presence of succinic dehydrogenase. Sodium malonate was employed as a blocking agent.

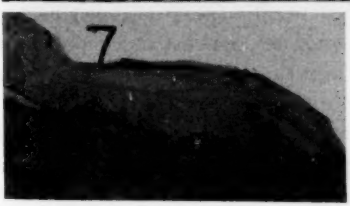
Rinehart's method and alcian blue combined with the periodic acid–Schiff technique were used for the determination of acid mucopolysaccharides.[15, 16] The former method involved staining in a colloidal iron solution for 40 minutes; the latter method utilized a 0.1 per cent alcian blue solution in 3 per cent acetic acid for 30 minutes and the periodic acid–Schiff reagent as a counterstain. As a further test for acid mucopolysaccharides, toluidine blue and azure A were used for demonstrating metachromasia in all tissues employing a 0.1 per cent solution.[17]

RESULTS

Glycogen.—Glycogen was demonstrated in the mucous membranes of both rats and hamsters in varying amounts (Figs. 1–8). The Hotchkiss reaction showed it to be present in the prickle-cell layer (stratum spinosum) of the stratum Malpighii and not in the stratum basale or stratum corneum. Glycogen was present in the 1-day-old animal (Fig. 18) and the greatest concentration was found in the 8-day-old rat and 42-day-old hamsters (Figs. 1, 2). Faint traces of glycogen were occasionally detected in 540-day-old animals and older (Figs. 3, 4). Differences in the observed glycogen concentration within any age group exceeded differences observed when comparing different age groups. The maximum intensity observed for 8-day animals was never observed in tissues of the ten 800–900-day-old animals. Localization was found to be most pronounced in sections of tissue taken from the palate of rats and hamsters and pouches of hamsters (Figs. 3, 6, 8). Little or no glycogen was found in the crevicular







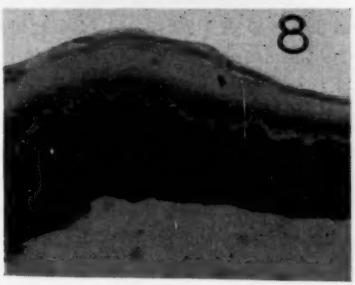


FIG. 1.—Cross-section of palate of 8-day-old rat stained by Hotchkiss PAS method for glycogen. Note intense localization of glycogen in prickle-cell layer. × 100.

FIG. 2.—Cross-section of palate of 42-day-old hamster stained by Hotchkiss PAS method for glycogen. ×100.

FIG. 3.—Cross-section of hamster cheek pouch of 540-day-old hamster. Note glycogen in the prickle-cell layer. Hotchkiss. ×100.

FIG. 4.—Diastase control of hamster cheek pouch stained by the Hotchkiss PAS technique. Note absence of glycogen.

FIG. 5.—Maxillary gingiva of 450-day-old hamster stained by Hotchkiss PAS method for glycogen. Note concentration of glycogen in oral surface of crestal gingiva. This tissue is free of inflammation. ×100.

FIG. 6.—Sagittal section of palate of 450-day-old hamster stained by Hotchkiss PAS technique. Note glycogen in epithelium. ×100.

FIG. 7.—Diastase control for Fig. 8. Note absence of glycogen. Hotchkiss. × 100.

FIG. 8.—Cross-section of palate of 8-day-old rat, littermate of animal in Fig. 1. Note that there appears to be less glycogen than in Fig. 1. Hotchkiss. × 100.

gingiva, while heavy concentrations were observed in well-keratinized crestal gingiva (Fig. 5). The periodic acid–Schiff method, as well as the Best carmine method, failed to demonstrate the presence of glycogen in the tissues of the monkey. The tissues for the most part were free of inflammation. Since these tissues were handled in exactly the same manner as the rodent tissues, it would appear that a species difference exists either in the concentration of glycogen or in the tissue reaction to the fixative.

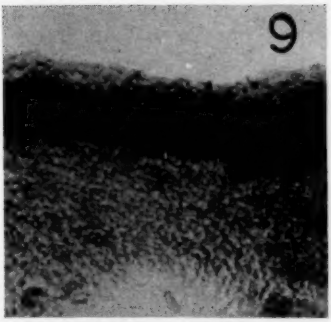
PAS-positive background material was found to be present in the tunica propria of both rats and hamsters. An increase in intensity of the staining reaction was observed with increasing age of the animals, being more pronounced in rats than in hamsters. This was probably due to an increased density of collagen, as sections stained by the Bauer reaction, which does not stain collagen, failed to reveal this age change. A moderately positive PAS stain, which became darker when combined with alcian blue, was distinguished in the intercellular cement of the epithelium. The basement membrane stained a deep red and became more distinct in the older animals of both species (Figs. 3, 5). A pale-pink reaction was observed in the prekeratin area of the epithelium and in the intercellular cement.

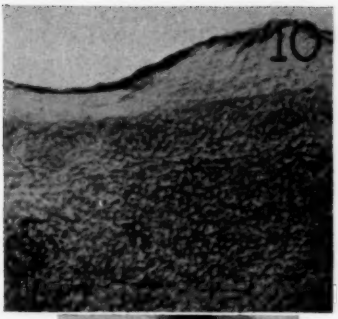
Succinic dehydrogenase.—Succinic dehydrogenase was localized predominantly in the basal layer and in two or three of the deeper layers of the stratum Malpighii (Figs. 9–12). Enzyme activity was noted in the epithelium of the crevicular, crest, and attached gingiva and in all parts of the palate. No age change was observed. In a few 1-day animals a diffuse pale staining reaction was observed in the Malpighii and corneal layers. However, this reaction was not confirmed when tissues from littermates of the same age were stained for succinic dehydrogenase. When succinic dehydrogenase localization of the gingiva taken from the 3-year-old rhesus monkeys was compared with that of the 42-day-old rodents (Figs. 13, 14), no differences were observed.

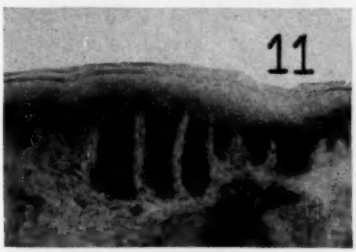
Acid mucopolysaccharides.—Definite localizations were found in the distribution of mucopolysaccharides stained by Rinehart's technique in the tunica propria of the gingival tissues. A pale-blue diffuse staining reaction just below the epithelium was noted in the tunica propria of the 1-day-old animals (Fig. 15). As the ages of the animals increased, an intense, localized, less diffuse reaction was observed deeper in the tunica propria (Figs. 16, 17). In the palatal tissues the distribution seemed to be more pronounced in the connective tissue of the rugae and was not present in any great quantity in the posterior region of the palate.

Alcian blue combined with periodic acid–Schiff was employed as a second method for the localization of acid mucopolysaccharides. In the 1-day-old animal a bluish diffuse stain was observed in the tunica propria immediately below the epithelium (Fig. 18). In 42–63-day-old rats and 850–900-day-old animals a darker, more diffuse reaction was observed (Fig. 19). The reaction increased in intensity from 1 to 42 days. Thereafter there was little change. In the old hamster (540 days) large patches of blue-staining material were observed in the tunica propria, being most pronounced in the rugae of the palate (Fig. 17).

Toluidine blue and azure A were used separately as a third method for detecting acid mucopolysaccharides. The localization in the tunica propria was similar to that observed by the Rinehart method (Figs. 16, 17). With non-buffered toluidine blue in distilled water at pH 5.6, the intercellular substance of the epithelium stained in the 1-day-old animal and failed to stain with increasing age. This could perhaps be







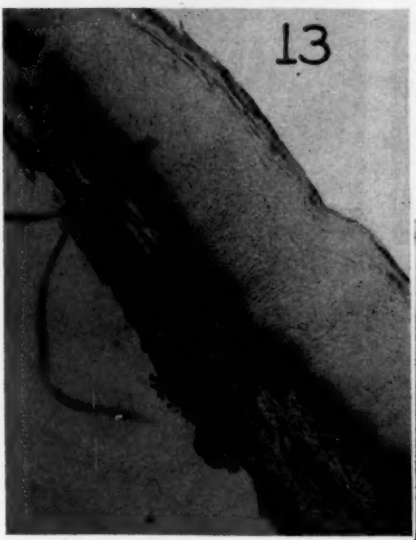


FIG. 9.—Section of palate of 26-day-old rat. Dark band in epithelium indicates succinic dehydrogenase activity. Blue tetrazolium. ×100.

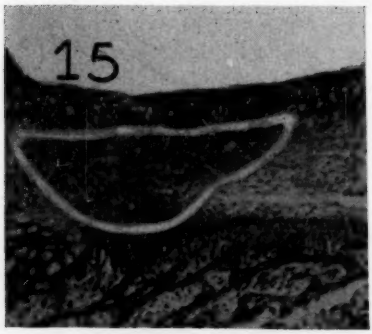
FIG. 10.—Control section for Fig. 9 incubated with sodium malonate which blocks the staining reaction for succinic dehydrogenase. Tetrazolium. ×100.

FIG. 11.—Section of palate of 26-day-old rat stained for succinic dehydrogenase with neotetrazolium. Note localization in stratum basale and in the lower part of prickle-cell layer. Tetrazolium. ×100.

FIG. 12.—Distribution of succinic dehydrogenase in the lower Malpighii layer of lower lingual gingiva of young rat. Neotetrazolium. ×100.

FIG. 13.—Localization of succinic dehydrogenase in the crevicular and marginal palatal gingiva of a young monkey. Neotetrazolium. ×100.

FIG. 14.—Control monkey palatal gingiva. Blocking was accomplished by sodium malonate. Neotetrazolium. ×100.



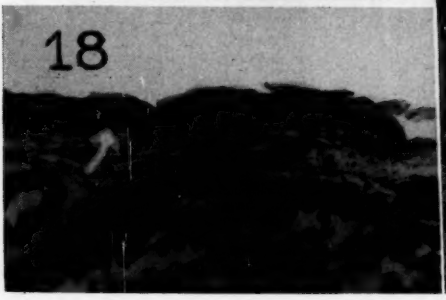


FIG. 15.—Upper jaw of 1-day-old rat, demonstrating a diffuse blue-staining reaction in the tunica propria. Rinehart's stain for acid mucopolysaccharides. ×100. The blue-stained areas are outlined in white for Figs. 15, 16, 17.

FIG. 16.—Palatal gingiva of 800-day-old rat, revealing intense area of localized blue stain rather than diffuse localization, as compared with the 1-day-old animal. Rinehart. ×100.

FIG. 17. Palatal rugae of 540-day-old hamster, showing intense localized blue-staining area in tunica propria. Rinehart. ×100.

FIG. 18.—Lower jaw of 1-day-old rat stained for acid mucopolysaccharides by the combined alcian blue–PAS method. Pale bluish diffuse stain, indicated by arrow, immediately below the epithelium is present in the tunica propria. Note alcian blue–PAS-positive material in the parakeratotic zone. There is little glycogen in the prickle-cell layer of the epithelium. Mowry. ×100.

FIG. 19.—Maxillary gingiva of 850-day-old rat, with a heavier reaction in the tunica propria. Mowry. ×100.

explained by the fact that in the 1-day animal the intercellular cement-like substance is not yet fully differentiated. Very weak gamma metachromasia was visible in the tunica propria at all ages, but distinct metachromasia was not perceptible in the intercellular cement. This would seem to indicate, in agreement with Thonard,[8] the presence of mucopolysaccharides but does not verify the presence of acid mucopolysaccharides.

DISCUSSION

The finding of increased glycogen in the crestal gingiva and palate when they were well keratinized is suggestive of a positive correlation between glycogen and keratinization. This positive correlation may exist between the rate of keratinization rather than the quantity of keratin. Since the palatal tissues and the oral surface of the crestal gingiva are presumably subjected to abrasion during mastication, a greater turnover of keratin may be required in these areas. This increased rate of keratinization may be associated with the observed glycogen deposition. It has previously been reported[18] that a mild injury to the stratum corneum of the skin produces an increase in glycogen. A great deal of variability in the intensity of glycogen localization was noted both in the same tissues of different animals (littermates) of the same age and in different sections of the same tissue. This variability could be due, as in the case of sulfhydryl,[19] to local metabolic alterations or to the lability of the glycogen. It has been shown by Turesky, Glickman, and Fisher[4] that the glycogen concentration can vary in the areas of the same section in relation to local areas of inflammation. The fact that our sections were generally free from inflammation and still contained appreciable amounts of glycogen indicates that gluconeogenesis is not essential for the production of substantial amounts of glycogen in the gingival epithelium, as suggested by Dewar.[6]

According to the observations presented, there appears to be some indication of differences between epithelial intercellular material and the ground substance of connective tissue. It was found in this investigation that the intercellular cement substance is both PAS-positive, alcian blue–positive, beta- to gamma-metachromatic, and Rinehart-negative; while the ground substance is weakly gamma-metachromatic, Rinehart-, alcian blue-, and PAS-postitive. The observation that Rinehart-positive material was most abundant in the rugae of the palate is suggestive of a function in maintaining the elevation of the rugae.

It has been previously pointed out by Shimizu[20] that low succinic dehydrogenase activity is related to glycogen deposition in neural structures. Wislocki, Fawcett, and Dempsey[10] and Bradfield[3] suggested that the glycogen deposition was correlated with a poor oxygen supply or a high anaerobic metabolism. Therefore, it was inferred that an intense anaerobic glycolysis in the neural structures produced the observed glycogen deposition. However, Braun-Falco[21] reported that he found both glycogen and succinic dehydrogenase activity in the outer root sheath of the hair.

In the oral mucous membrane, succinic dehydrogenase activity was found to remain constant with age by histochemical methods. The localization in the basal cells indicates an increased amount of aerobic glycolysis, as compared with the remainder of the epithelium. Serial sections stained for glycogen with the PAS method using diastase controls indicated that the oxidation-reduction system in oval mucous membrane

containing succinic dehydrogenase was diminished in the areas containing glycogen. This would indicate that another of the oxidative systems or an anaerobic system was utilized in the metabolism of glycogen.

The consistent finding of succinic dehydrogenase in all the tissues studied indicates the importance of this particular enzyme in the vitality of living cells. It is interesting to note that the distribution of succinic dehydrogenase in the oral mucous membrane is apparently identical with the distribution of mitosis as reported by Hirt, St. Hartl, and Muhlemann.[22]

SUMMARY

Glycogen in the epithelium of the oral mucosa of rodents was found to decrease in concentration with age and was found to be abundant in areas where no inflammation was present. Glycogen was not demonstrable in the monkey mucosa.

A possible correlation between glycogen deposition and rate of formation of keratin in rodents is suggested by its presence in greater amounts where tissue abrasion during mastication may occur, such as the palate and oral surface of the crestal gingiva.

The maximum succinic dehydrogenase activity was observed in the basal cells, with diminishing activity in the deeper layers of the stratum Malpighii. The distribution was the same in all the animals studied and was not affected by age. Glycogen was found to be most abundant in areas of diminished succinic dehydrogenase activity in the rat and hamster. Since succinic dehydrogenase and glycogen are not found in the same location, the possibility exists that other enzyme systems are associated with glycogen formation.

Material which showed a positive reaction for acid mucopolysaccharides with the Rinehart technique increased with age and was most pronounced in the tunica propria of the palatine rugae of the rodents.

Epithelial intercellular cement was found to be alcian blue–PAS-positive and Rinehart-negative, while the ground substance of the tunica propria was alcian blue–, PAS-, and Rinehart-positive. This suggests a difference in the mucopolysaccharide composition of these tissues.

REFERENCES

1. Eichel, B., and Swanson, A. A. *Intracellular Oxidative Enzymes: Succinic Dehydrogenase, DPN Cytochrom C, Reductase, Cytochrome Oxidase, and Catalase in Oral, Liver, and Brain Cortex Tissues.* Air Univ. School of Aviation Med. U.S.A.F., June, 1956.
2. Person, P., and Burnett, G. W. Dynamic Equilibria of Oral Tissues. II. Cytochrome Oxidase and Succinoxidase Activities of Oral Tissues, *J. Periodont.*, **26**:99, 1955.
3. Bradfield, J. R. G. Glycogen of Vertebrate Animals, *Nature*, **167**:40, 1951.
4. Turesky, S., Glickman, I., and Fisher, B. The Effect of Physiologic and Pathologic Processes upon Certain Histochemically Detectable Substances in the Gingiva, *J. Periodont.*, **30**:116, 1959.
5. Weiss, M. D., Weinmann, J. P., and Meyer, J. Degree of Keratinization and Glycogen Content in the Uninflamed and Inflamed Gingiva and Alveolar Mucosa, *J. Periodont.*, **30**:208, 1959.
6. Dewar, N. R. Observations on the Composition and Metabolism of Normal and Inflamed Gingivae, *J. Periodont.*, **26**:29, 1955.
7. Trott, J. R. An Investigation into the Glycogen Content of the Gingiva, *D. Practitioner*, **7**:234, 1957.
8. Thonard, J. C., and Scherp, H. W. Characterization of Human Gingival Epithelium Intercellular Cementing Sustance, *J. D. Res.*, **38**:711, 1959 (abstr.).
9. Engel, N. B. Water Soluble Muco-Proteins of the Gingiva, *J. D. Res.*, **32**:779, 1953.
10. Wislocki, G. B., Fawcett, D., and Dempsey, E. Staining of Stratified Squamous Epithelium of

Mucous Membrane and Skin of Man and Monkey by the Periodic Acid Method, *Anat. Rec.*, **110**:359, 1951.

11. CASTENFELT, T. *Toothbrushing and Massage in Periodontal Disease.* Stockholm: Nordisk Rotogravyr, 1952.
12. HOTCHKISS, R. D. Microchemical Reaction Resulting in Staining of Polysaccharide Structures in Fixed Tissue Preparations, *Arch. Biochem.*, **16**:131, 1948.
13. PADYKULA, HELEN A. The Localization of Succinic Dehydrogenase in Tissue Sections of the Rat, *Am. J. Anat*, **91**:107, 1952.
14. FORMISANO, V. R., and MONTAGNA, W. Succinic Dehydrogenase Activity in the Skin of the Guinea Pig, *Anat. Rec.*, **120**:893, 1954.
15. RINEHART, J. F., and ABUL-HAJ, S. K. Improved Method for Histological Demonstration of Acid Mucopolysaccharides in Tissue, *Arch. Pathol.*, **52**:189, 1951.
16. MOWRY, R., and WINKLER, C. H. The Coloration of Acid Carbohydrates of Bacteria and Fungi in Tissue Sections with Special Reference to Capsules of *Cryptococcus neoformans, Pneumococcus,* and *Staphylococcus, Am. J. Pathol.*, **36**:628, 1956.
17. LILLIE, R. D. *Histopathologic Technique: Practical Histochemistry.* New York: Blakiston Co., Inc., 1954.
18. LOBITZ, E. C., JR., and HOLYOKE, J. B. The Histochemical Response of the Human Epidermis to Controlled Injury: Glycogen, *J. Invest. Dermatol.*, **22**:189, 1954.
19. BARRNETT, R. J., and SELIGMAN, A. M. Histochemical Demonstration of Sulfhydryl and Disulfide Groups of Protein, *J. Natl. Cancer Inst.*, **14**:769, 1954.
20. SHIMIZU, N. Histochemical Studies of Glycogen of the Area Postrema and the Allied Structures of the Mammalian Brain, *J. Comp. Neurol.*, **102**:323, 1955.
21. BRAUN-FALCO, O. *The Biology of Hair Growth.* Edited by W. MONTAGNA and R. A. ELLIS, chap. 4. New York: Academic Press, 1958.
22. HIRT, C. M., ST. HARTL, and MUHLEMANN, H. R. The Distribution of Mitoses in the Epithelium of the Interdental Papillae of the Rat Molar, *J. Periodont.*, **26**:229, 1955.

Standardized Paper Electrophoresis of Parotid Saliva

A Study of Variables in Technique and Flow Rate as Factors in Resolution

SHELDON HOLEN, NORMAN HABER, and IRMA TUCK-WEISS

Murry and Leonie Guggenheim Foundation, Institute for Dental Research, New York University College of Dentistry, New York

The value of paper electrophoresis, so simple and rapid, has been established as a diagnostic tool in clinical medicine.[1] Extensive studies with blood serum[2] have shown that the distribution of blood proteins is quite uniform and that the reproducibility of individual patterns is very good. On the other hand, few investigations have been reported for saliva, whole, mixed, or the separate secretions. These show variations in composition. In contrast to blood, salivary proteins are in low concentration (0.2–0.4 per cent as against 6–7 per cent), and our knowledge of their origin and composition is much more fragmentary. Although little is known of their role in systemic or dental conditions, a standardized technique with paper electrophoresis would be valuable in adding to this information.

Our investigations of salivary electropherograms were initiated to establish a routine procedure under standardized operational conditions. Patterns of parotid saliva from individuals under controlled stimulation may then be compared and evaluated. The apparent criterion of optimum resolution reported by previous investigators is reflected in the variations of the number of zones (2 to 12) reported. Ferguson, Krahn, and Hildes[3] reported a total of 9 bands but indicated that not all these bands appeared in patterns of all subjects. Kinersly[4] and Kinersly and Leite[5] made the same observation but never obtained 9 components. Gabl and Egger[6] showed 5 to 7 components. Drevon and Donikian[7] reported 4 or 5 components, the number varying with different buffer systems. Conversely, the presence of no more than 2 or 3 bands is often interpreted as evidence of poor resolution. Hoerman,[8] dissatisfied with paper as a supporting medium, demonstrated 12 zones in a borate buffer on a starch gel. Weinstein, Heskins, and Jackson[9] pointed out that conditions designed for serum analysis were not desirable for parotid saliva. Though they reported 11 components, the frequency of occurrence of this number was implied but not shown. Of the several groups of independent investigators, Ferguson, Krahn, and Hildes[3] showed some differences in pattern resolution from the same individual for samples obtained at different flow rates. They reported an increase in the total protein content, as well as an increase in the electrophoretically separable components when the flow rate increased from 0.05 to 1.26 ml/min in a limited number of cases.

This work was supported by U.S.P.H.S. research grant D-268 from the National Institute of Dental Research, National Institutes of Health.

Received for publication August 26, 1960.

It seemed important, therefore, first to check the influence of operational factors and then with a standardized procedure to determine the characteristics of parotid saliva as they are dependent upon flow rate. Only under these conditions are clinical evaluations valid. The present study was undertaken to standardize the operational factors of paper electrophoresis with an instrument* readily available for diagnostic laboratories. Veronal buffer at pH 8.6 and $\mu = 0.05$ was chosen to permit comparison of salivary patterns with blood serum patterns. Using the standardized experimental conditions, patterns from the same and different individuals were compared for optimum resolution at mild and hyperstimulation levels.

MATERIALS AND METHODS

The subjects were 50 healthy adults of the staff and student body. The samples of parotid saliva were obtained by means of an acrylic, double-walled suction cup† placed over the opening of one parotid gland duct. The flow was induced by a single, repeated, or prolonged stimulation with lemon juice concentrates or fresh lemon. Stimulation was defined by flow rate: (1) mild stimulation—obtained by swabbing the mouth with dilute lemon juice, or by smoking, and yielding under 1 ml/min of parotid saliva from one parotid gland; (2) hyperstimulation—obtained by sucking on a fresh lemon or drinking fresh lemon juice, and yielding a flow rate greater than 1 ml/min. All samples, varying in volume from 8 to 10 ml. were collected at room temperature and stored at 4° C. until ready for processing.

Five-ml. samples were placed in cellophane casings‡ (25/64 in.) and dialyzed against distilled water (1 liter/sample) at 4° C. for periods of time varying between 12, 24, and 48 hours. In addition, some samples were dialyzed against veronal buffer. Other samples were run without any dialysis.

Following this treatment, concentration of the samples was effected by direct pervaporation at room temperature, using a fan. The casings were kept under tension by means of a tightly wound rubber band at the lower portion of the vertically suspended tubing. As the evaporation progressed, the rubber band was moved upward, entrapping the remaining liquid. With the small-sized casing described, the concentration factor was conveniently controlled. In these studies about a tenfold concentration was readily obtained in 3–5 hours.

Both a striper and a micropipette were used to apply the sample to the paper strip. To the 3-cm. paper (S and S 2043A gl), supplied with the instrument, the application with the striper was a double operation and resulted in the sample's spreading at the outer edges. On the other hand, a conical-tipped ultra-micropipette, carefully fire-polished, delivered a 20-lambda sample in a single operation. By lightly touching the tip of the pipette about 7–8 mm. from the paper's edge, the sample (20 lambda) was applied to the strips, stretched taut on the support. This process was repeated, working toward the center and then out to the other edge. After a uniformly oval spot was attained, the remainder of the sample was delivered into the center of the zone.

* Spinco Model R Electrophoresis Unit, Beckman Instruments, Spinco Division, Belmont, California.

† These cups were made in quantity with acrylic resin, polyethylene tubing, and 15-gauge hypodermic needles, using a Lashley cup as a model.

‡ The casings were obtained from Visking Co., Chicago, Illinois, Division of Union Carbide.

To minimize sample spreading, the direct pipette method was always used on dry strips. To insure uniform distribution of buffer along the whole strip, it was found adequate to flush down both sides of the supported strips with buffer, using a hypodermic syringe. The stream of buffer was directed across the width of each strip, approximately 3–5 cm. away from the application area on both sides. About 10–15 ml. of buffer were sufficient for all eight strips in a cell. The entire operation was completed in about 1 minute. The cell cover was installed and the power supply connected as soon as the buffer level rose to the zones of application.

The power supply used* is a regulated source and has fixed characteristics. The variability of the regulated output is low and was checked and readjusted during the first half-hour of the 17-hour run. What is more important is that the regulation applies either to the voltage or to the amperage, but not to both simultaneously. To achieve control of this non-regulated variable, a 150,000-ohm 2-watt "ohmite" potentiometer was installed in series with the red (positive) terminal of the power unit cell plug. Using this device, along with the control knob on the power supply, simultaneous control of both voltage and current levels was obtained within the limits of the system. Using a single sample, repeated runs were made to determine the pattern formation at 85, 125, 180–200, 300, 400, and 500 volts at two current levels. From this study, the most satisfactory electrical conditions were chosen and checked for reproducibility. Subsequent determinations were always run for 17 hours at 300 volts and 4–4.5 ma., the selected optimum.

After electrophoresis, the strips were immediately transferred to a preheated oven at 120° C., dried for half an hour, and then stained. Of the various dye methods tried, two were singled out for use. Routinely, the strips were allowed to soak several hours in a 0.02 per cent solution of Nigrosine (water-soluble) in 5 per cent aqueous acetic acid. A water rinse removed much of the excess dye, and a short wash in dilute sodium hypochlorite† solution followed by a water rinse removed the background color. A 10 per cent acetic acid rinse (5–10 min.) can alternately be used to lighten the background color.

A saturated solution of Buffalo Black NBR‡ in 10:1 methanol acetic acid solution, to which 5 per cent zinc acetate was added, was also used. A series of nine rinses, of decreasing dye concentration in methanol, with pure methanol as the last rinse, gave sharp blue bands on a pale blue background. Only 10 minutes in the initial dye solution were required. A pure white background can be obtained by rinsing in 50 per cent aqueous acetic acid solution or by the "clorox" bleach method described above.

RESULTS

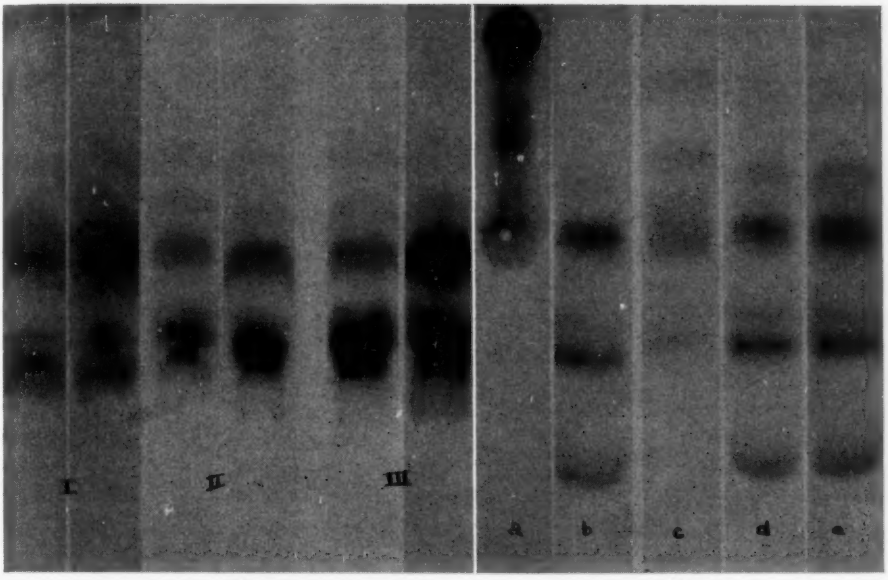
In the buffer system we studied, the number, mobility, and intensity of well-defined electrophoretic components were affected by variations in the following technique factors: (1) dialysis treatment, (2) sample application, (3) voltage-current conditions, and (4) staining procedure. Figure 1 illustrates in part (*A*) the poorly resolved, in-

* Spinco "Duostat" recommended for use with the model R unit, Beckman Instruments, Spinco Division, Belmont, California.

† Commercial "Clorox" diluted *ca.* 1 part to 2000–3000 times.

‡ Buffalo Black NBR purchased from National Aniline and Chemical Co.

tensely stained patterns for undialyzed samples. In part (*B*) a comparison of patterns for a sample, undialized and dialyzed 48 hours, shows the loss of a component resulting from prolonged dialysis. The good reproducibility of replicate runs is also evident. Figure 2 shows patterns developed by the two methods of sample application—pipette and striper. These determinations were made with dialyzed concentrates of sample previously run without dialysis, as shown in Figure 1 (*A*). The reproducibility of the pipette application technique should be noted in the duplicates illustrated, though these samples were run in quadruplicate. Some dissimilarity between the patterns developed by each technique may be recognized.



A *B*

FIG. 1.—(*A*) Repeated runs with three different undialyzed samples. (*B*) A sample which fractionated with dialysis: (*a*) serum, (*b*) undialized sample, (*c*) sample dialized for 48 hours (note loss of band), (*d*) and (*e*) repeated runs of undialyzed sample.

In Figure 3 there can be seen the alterations in the patterns which result from changes in voltage-current conditions. In parts (*A*) and (*B*), we note the increase in mobility and the concurrent limitations imposed by increased amperages. In part (*C*) the effect of voltage increases at constant current levels was noted.

From these combined studies, we adopted a standardized procedure for all subsequent determinations. In preparation for electrophoresis, a sample was dialyzed against distilled water overnight at 4° and pervaporated to 8–10 times its original volume. A 20-lambda aliquot of this concentrate was applied to a paper strip with a micropipette and processed for 17 hours at 300 volts (constant) at 4–4.5 ma. The pattern was developed with Nigrosine stain. Under these conditions, Figures 4 and 5 illustrate the patterns obtained at two different levels of stimulation previously defined. For the 30 subjects from which parotid saliva was obtained with mild stimulation, an average of 10 ml. of saliva was collected at a flow rate of from 0.28 to 0.70 ml/min. Fifteen of these are shown in Figure 4. In the group under hyperstimulation, an average of 10 ml.

of saliva was collected at flow rates of from 1 to 5 ml/min. The results for 15 of these subjects are illustrated in Figure 5. The differences in the number, mobility, and apparent concentration of electrophoretic components (intensity of stain) is observable.

DISCUSSION

In standardizing the operational technique, we repeatedly used a single individual sample to observe changes or reproducibility. Eight to 10 ml. of parotid saliva obtained by a single stimulation with lemon juice gave a sufficient quantity of concentrate to

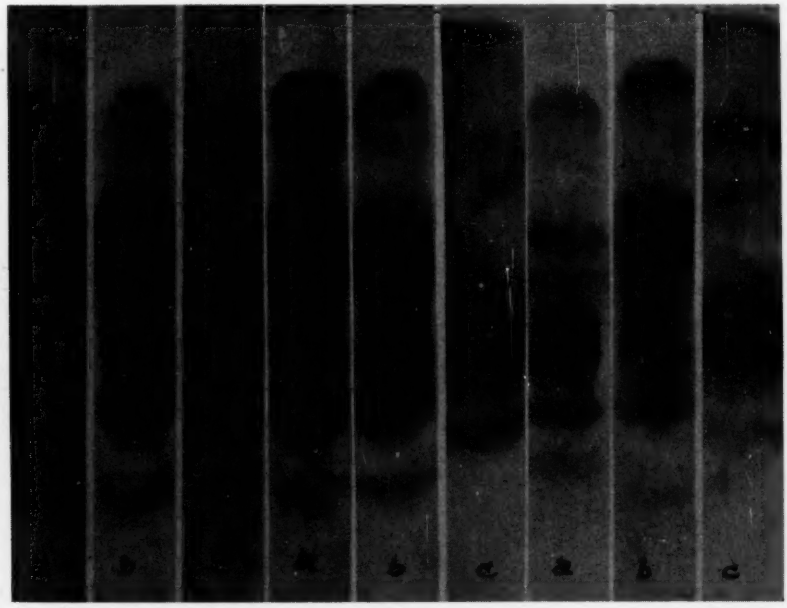
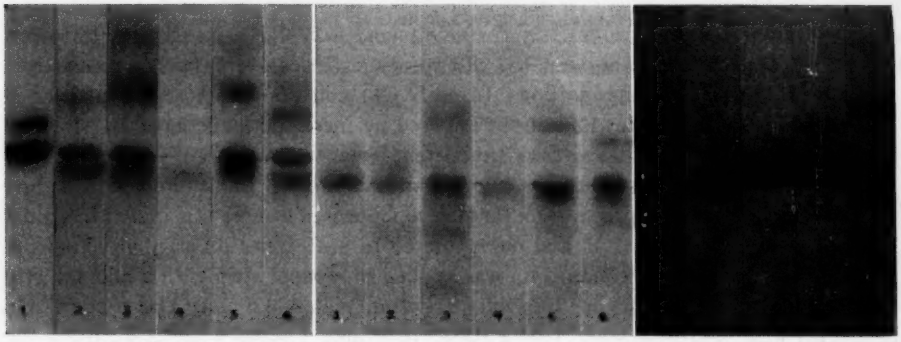


FIG. 2.—Sample from Fig. 1 (*A*) after overnight dialysis. (*a*) and (*b*) are repeated runs of pipette application of sample, (*c*) shows the stripper application of the sample.



A *B* *C*

FIG. 3.—(*A*) Effect of current and voltage (constant) for a single sample: *1*, 200 volts, 1–2.5 ma.; *2*, 200 volts, 5–5.5 ma.; *3*, 300 volts, 4–4.5 ma.; *4*, 300 volts, 8–9 ma.; *5*, 500 volts, 4.5 ma.; *6*, 500 volts, 9–13 ma. (*B*) Another sample run as Fig. 3 (*A*). (*C*) Effect of current (constant) and voltage for a single sample: *1*, 3 ma., 85 volts; *2*, 1.25 ma., 200 volts; *3*, 2.5 ma., 200 volts; *4*, 5 ma., 200 volts; *5*, 5 ma., 400 volts (note reverse polarity).

carry out 15–20 replicate determinations. Although some salivas may occasionally be encountered which will give good electrophoretic patterns without dialysis, for a routine procedure, dialysis is preferred. In general, undialyzed samples showed poor resolution and reduced migration but were intensely stained. Prolonged dialysis (24–48 hr.) against distilled water gave patterns with the intensity of the stained zones generally reduced. In addition, dialysis against veronal buffer did not improve the results. Therefore, before concentration, dialysis overnight insures optimum resolution with a minimum loss of dye-staining components.

Although Kinersly and Leite[5] reported some fractionation with unconcentrated samples, we favor the use of a concentration of 8–10 times to insure defined patterns.

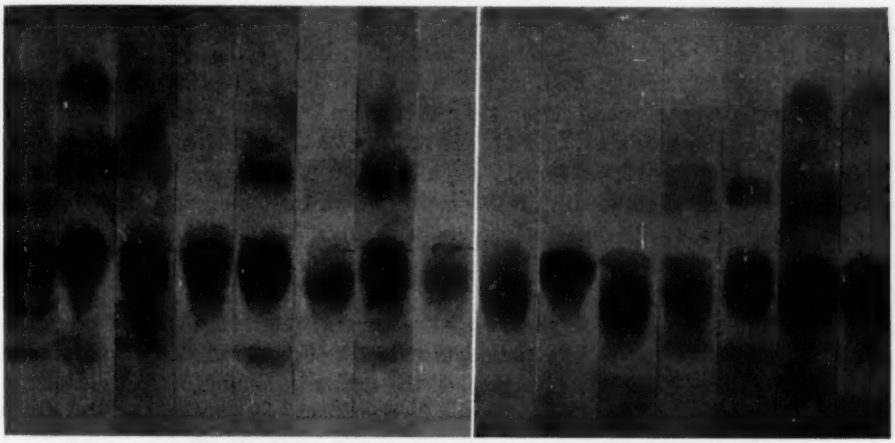


FIG. 4.—Typical patterns obtained with flow rates under 1 ml/min (mild stimulation)

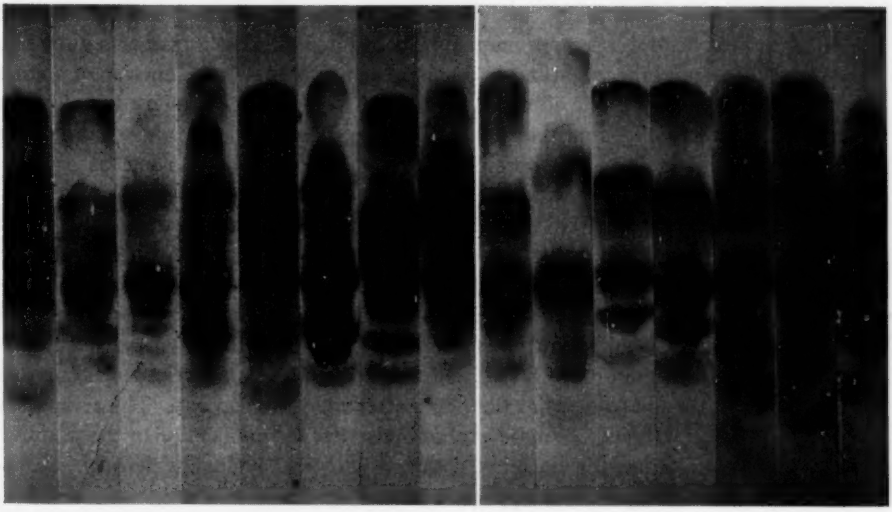


FIG. 5.—Typical patterns obtained with flow rates greater than 1 ml/min (hyperstimulation)

Samples initially clear often remain clear after concentration, whereas others tend to become somewhat turbid. This turbidity is not necessarily assumed to be the primary result of denaturation but may indicate differences in the solubility of salivary constituents. Concentration by pervaporation seemed entirely satisfactory. For a significant comparison of paper electropherograms for diagnostic purposes, it seemed evident to us that a standard procedure for processing was necessary before electrophoresis. We adopted overnight dialysis and concentration by pervaporation as a routine operation. Our results shown in Figures 1 and 2 support this contention.

In evaluating the two methods of sample application, we noted that the striper gave less broad bands. With pipette application, the resolution appeared better, all other conditions being equal and optimum. Furthermore, with this method, the patterns appear characteristic for individual salivas. The uniformity of this individualistic pattern is shown in the series of repeated runs illustrated in Figure 2. It must be pointed out that excellent patterns were obtained with the striper when wider paper was used. Unfortunately, the electrophoresis unit used in this study is not constructed to accommodate conveniently strips of paper wider than 3 cm.

In the illustrations (Figs. 1 and 2), the two dye techniques have been shown, and both appear equally effective. Buffalo Black NBR gives a permanent record, whereas Nigrosine will fade slightly with time. Some variations in staining with Buffalo Black NBR were observed with different batches of the dye. The intensity of the resolved zones differs with both dyes, the Nigrosine appearing to be more sensitive in over-all staining.

The Nigrosine stains the salivary material a blue-gray color. However, an interesting zone has been noticed at or near the origin in strips of most samples. It is seen as a Nigrosine-repellent white spot overlapping the regularly staining salivary material. This observation is entirely absent with the Buffalo Black NBR technique. The nature of this component has not yet been ascertained or previously reported.

The alteration in patterns induced by variations in the voltage-current conditions has been shown in Figure 3. We noted the increase in mobility and the concurrent limitations imposed by increased amperages. An increase in the current from 3 to 13 ma. enhanced the heating effect, promoted evaporation, and therefore changed the pattern. We also observed the variations in mobility and resolution as a function of voltage at constant current levels. The apparent reversal of the direction of migration of one of these zones is illustrated. From these studies, the optimum level of voltage and current for the best reproducible results were chosen as 300 volts and 4–4.5 ma. at constant voltage. These conditions were adopted for routine work.

The total procedure evolved was then applied to the analysis of parotid saliva samples obtained from the two groups of subjects differing in stimulated flow rate. Many of the samples collected under hyperstimulation were clear, while saliva collected over a longer period of time more often appeared cloudy. This was associated with periodic slippage of the cup from the duct opening, requiring that it be replaced. This cloudiness was either spun down or allowed to settle. Subjects experienced no pain or discomfort during or following collection, no matter what the flow rate. The actual flow was not constant, greatly increasing several seconds after the application of the stimulus.

With mild stimulation, the patterns obtained showed varying degrees of electrophoretic separation (Fig. 4). For approximately 70 per cent of this group, the bands

obtained showed little or no mobility. In the remainder of the group a wide range of patterns was observed.

Electrophoretic fractionation was regularly observed in patterns of samples obtained by hyperstimulation, as shown in Figure 5. The constancy of replicate runs with this type of sample has been noted (Fig. 2). There is an obvious alteration in the electrophoretic pattern obtained with parotid saliva, depending upon the rate of salivary flow commensurate with stimulation. Since the technique used was standardized and known to be reproducible, there is little doubt that the differences observed can be attributed to the rate of flow. The increased intensity of staining in the high flow-rate group indicates a generally increased organic output concurrent with hyperstimulation. In harmony with this, an increased viscosity was often noted in such samples.

It is tempting to suggest that, under stimulation, the functioning of the parotid gland may be reflected in the number and distribution of the electrophoretic components. If this is so, it is not difficult to understand the wide variation in reports of the number of components of parotid saliva given by many previous investigators. Because of the good reproducibility of the results in the present study, it is expected that we can establish a reference system either for comparison or for independent quantitation of the patterns. This information may then be valuable for significant diagnostic or clinical purposes.

SUMMARY

A routine procedure for paper electrophoresis has been developed for parotid saliva in veronal buffer, pH 8.6, μ, 0.05. Samples were dialyzed and concentrated by pervaporation, prior to analysis. Electrophoresis was carried out for 17 hours at 300 volts, 4–4.5 ma. as constant voltage, and the strips were stained with Nigrosine.

This standardized technique was checked for reproducibility to show the constancy of the number of fractions produced by electrophoretic resolution, the relative positions of these fractions in repeated runs of the sample, the total pattern picture characteristic of and obtained with each particular sample. While some slight variations due to staining were recognized, an entirely satisfactory reproducible technique has been evolved.

A study of 50 subjects has shown that a flow rate greater than 1 ml/min for a single parotid gland appears to be required for adequate electrophoretic studies. This factor appears to be related to gland function rather than to the conditions for electrophoretic analysis, already established as routine. Further investigations of these electrophoretic components are now in progress in our laboratory.

REFERENCES

1. Wall, R. The Use of Serum Protein Electrophoresis in Clinical Medicine, *A.M.A. Arch. Internal Med.*, **102**:618, 1958.
2. Sabotka, H., and Stewart, C. P. *Advances in Clinical Chemistry*. New York: Academic Press, 1959.
3. Ferguson, M. H., Krahn, H. P., and Hildes, J. A. Parotid Secretion of Protein in Man, *Can. J. Biochem. and Physiol.*, **36**:1001, 1958.
4. Kinersly, T. Preliminary Paper Electrophoretic Study of Saliva, *Yale J. Biol. and Med.*, **26**:211, 1953.

5. KINERSLY, T., and LEITE, H. B. Paper Electrophoretic Study of Some Components of Saliva, *Yale J. Biol. and Med.*, **29**:496, 1956–57.
6. GABL, F., and EGGER, E. Charakterisierung von Speichelproteinen, *Clin. Chim. Acta*, **4**:62, 1959.
7. DREVON, B., and DONIKIAN, R. Sur les protéines de la salive parotidienne humaine, *Compt. rend. soc. biol.*, **150**:1206, 1956.
8. HOERMAN, K. On the Zone Electrophoresis of Human Parotid Saliva in Starch Gels, *J. Lab. Clin. Med.*, **53**:64, 1959.
9. WEINSTEIN, E., HESKINS, D., and JACKSON, S. An Improved Technique for the Paper Electrophoresis of Parotid Saliva, *J. D. Res.*, **39**:110, 1960.

Whole Saliva, Salivary Sediment, and Supernatant as Fermenting Agents for Foods

DONALD J. BECK and BASIL G. BIBBY
Eastman Dental Dispensary, Rochester, New York

During the course of an investigation into the fermentation of foodstuffs by saliva and the development of a new method of measuring acid production in these mixtures,[1] the attention of the investigators was directed toward the relative fermentative capabilities of whole saliva, salivary sediment, and supernatant.

It had previously been shown by Sreebny, Kirch, and Kesel,[2] using pure sugars as substrate, that the intrinsic glycolytic activity of saliva lies in the micro-organisms, i.e., the enzymes are largely intracellular. This finding was confirmed by Hartles and Wasdell,[3] again with pure sugar substrates. In addition, these latter investigators reported that, despite the fact that the supernatant from centrifuged saliva showed practically no glycolysis per se, the glycolytic activity of salivary sediment in the absence of supernatant was reduced considerably compared with that of whole saliva. They offered no suggestion as to what the mechanism or factor in the supernatant might be, which, although having no intrinsic glycolytic powers, greatly increased glycolysis by the organisms.

In view of the marked variations we noted between fermentations by whole saliva, salivary sediment, and supernatant in our early foodstuff experiments and the findings of these authors with pure sugars, it was decided to make some investigation of the problem in the present study. This investigation was designed (*a*) to attempt to confirm the findings of these two groups of workers, using foods (the normal bacterial substrate in the mouth) rather than pure sugars, and (*b*) to make some investigation of the possible nature of the glycolytic adjuvant powers of salivary supernatant.

The so-called periodic neutralization technique,[1] using regular titrations with sodium hydroxide during the course of fermentation, was employed to measure the acid production in the various test mixtures.

EXPERIMENTAL METHODS

To compare whole saliva, salivary sediment, and supernatant, 5 per cent suspensions of chocolate cookie, salted cracker, corn flakes, chocolate ice cream, and banana were produced in a Waring Blendor. Three series of 30-ml. samples of these five food suspensions were prepared, and to each sample in the first series was added 10 ml. of whole, paraffin-stimulated, pooled saliva. For the other two series, whole saliva from the same pool was centrifuged for 30 minutes at 9000 rpm, sediment and supernatant

This investigation was supported in part by a U.S.P.H.S. research grant, D-607, from the National Institute of Dental Research, National Institutes of Health.

Received for publication September 6, 1960.

separated, and each made up to the original volume of the sample with distilled water. The second series employed 10 ml. of the suspension of salivary sediment thus formed in each sample, and the third series employed 10 ml. of the salivary supernatant. Controls consisting of distilled water plus whole saliva, distilled water plus sediment, and distilled water plus supernatant were set up. pH readings were taken for each sample, and they were then incubated at 37° C. for a total of 6 hours, with intermittent manual agitation. At the end of each hour of fermentation the pH was checked, and the mixtures were titrated back to the original pH with 0.1 N sodium hydroxide, using a pH meter* to check the end-point of the titration. The sum of the six hourly titrations was taken as the total acid production in the mixtures.

A 96-hour adaptation of this periodic neutralization technique with one food (salted cracker) was also employed to compare the fermentative capacities of whole saliva and sediment. Each day of the fermentation the samples were titrated at the end of the first, second, third, fourth, fifth, sixth, twelfth, and twenty-fourth hour. The fermentation mixtures consisted of 30 ml. of a 5 per cent food suspension with 5 ml. of saliva, or the sediment of 5 ml. of saliva, made up to 5 ml. with distilled water.

In an attempt to explain the reduced acid production observed when foodstuffs were fermented with salivary sediment, as compared with whole saliva fermentations, two properties of whole saliva were investigated—those of buffering activity and of amylase activity. Either one or both of these properties, being removed with the supernatant, might explain the reduced acid production from sediment.

A definite difference in buffering capacity between whole saliva and salivary sediment was demonstrated when 20-ml. samples of whole saliva, sediment, and supernatant were titrated, with regular 0.5-ml. increments, with 0.01 N sodium hydroxide. The pH was recorded after each increment. A similar process was carried out with another series of samples, using 0.01 N lactic acid. When the curves so obtained were plotted together, the buffering capacities of these three salivary samples could be demonstrated.

In order to test the hypothesis that reduced amylase or buffering activity might reduce acid production from sediment during fermentation, an experiment to check the effect of the addition of artificial amylase and buffers to salivary sediment was run. The test foodstuff used was salted cracker, in the proportion of 30 ml. of a 5 per cent suspension to 5 ml. of saliva or other fermenting agent. To give a basis for comparison, samples consisting of food plus whole saliva and food plus sediment suspended in normal saline were used. An amylase activity which theoretically should have approximated that of whole saliva was achieved with one sample by the addition of 5 mg. of amylase† to sediment suspended in saline. A test of the combined effect of artificial amylase and buffering was made by adding 5 mg. of amylase to sediment suspended in a "synthetic saliva" basically similar to the calcifying solution of Burger, Lavine, Deane, and Sobel.[4]

Further tests were made in this experiment to determine whether the low acid production by sediment could be accounted for by osmotic shock to the organisms by the suspension of the sediment in distilled water (a technique which had been used in the earlier experiments) or by a physical effect of centrifugation. To study the possibility

* Beckman Model M Glass electrode 43509.

† "Taka-Diastase," Parke, Davis and Co., Detroit, Michigan.

of osmotic shock to the cells, a sample composed of food plus sediment suspended in distilled water was set up, and, to test the theory that some physical effect during centrifugation might have been involved, another sample employed saliva that was reconstituted by remixing sediment and supernatant. Controls used in this experiment consisted of water plus whole saliva, water plus sediment, food plus amylase, and food plus buffer plus amylase.

RESULTS

The comparison of whole saliva, salivary sediment, and supernatant as fermenting agents is shown in Table 1 and Figure 1. The salivary fermenting agents plus water controls showed no significant change in pH. From these results it can be seen that whole saliva brought about the most rapid and complete acid production in all cases.

TABLE 1*

COMPARISON OF ACID PRODUCTION FROM FIVE FOODS FERMENTED FOR 6 HOURS WITH WHOLE SALIVA, SALIVARY SEDIMENT, AND SUPERNATANT —PERIODIC NEUTRALIZATION TECHNIQUE

	TITRATABLE ACIDITY (ML. 0.1 N NaOH)				
	Chocolate Cookie	Salted Cracker	Corn Flakes	Chocolate Ice Cream	Banana
Whole saliva.....	10.9	19.1	15.0	15.8	15.5
Salivary sediment....	2.3	3.3	0.6	5.4	0.8
Salivary supernatant..	2.1	3.9	1.8	3.9	2.5

* Each figure in this table represents the sum of six hourly titrations.

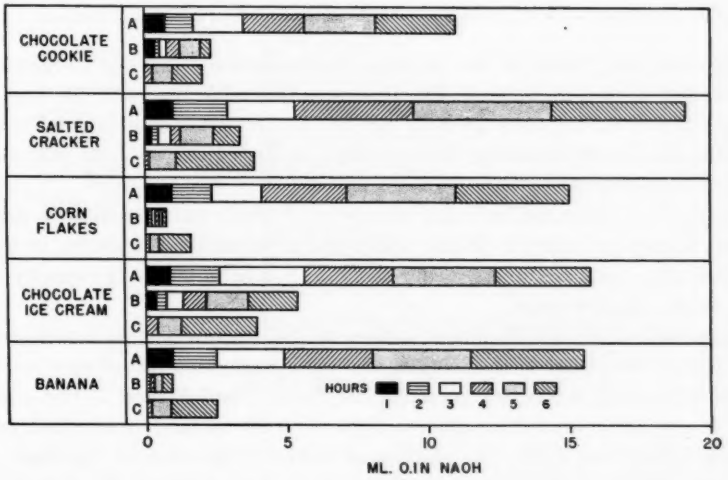


FIG. 1.—Relative effectiveness of whole saliva (*A*), salivary sediment (*B*), and salivary supernatant (*C*) in fermenting five representative foods. Acid production measured by the periodic neutralization technique with hourly titrations for a total of 6 hours.

The results achieved with salivary sediment as the fermenting agent show that, although acid production commenced simultaneously with sediment and whole saliva, considerably less acid was produced during fermentation with the former agent than with the latter. This suggests the presence of a glycolytic adjuvant in salivary supernatant.

During the first 3–4 hours of the fermentation with supernatant, little or no acid was produced from any of the substrates. This is in agreement with the results of earlier workers and, presuming that the enzymes are largely intracellular, is to be expected. During this time lag, however, proliferation of the few bacteria unavoidably left in the supernatant after centrifugation was occurring. In the later stages of fermentation, this increase in bacterial population was reflected in measurable acid in the culture. Once this period was reached, acid production in supernatant was greater than that in sediment, again suggesting that some stimulatory agent was removed as part of the supernatant in the production of sediment.

TABLE 2

ACID PRODUCTION FROM FERMENTATION OF SALTED CRACKER WITH WHOLE SALIVA AND SALIVARY SEDIMENT OVER 96-HOUR PERIOD—PERIODIC NEUTRALIZATION TECHNIQUE WITH EIGHT TITRATIONS EACH DAY

	Titratable Acidity (Ml. 0.1 N NaOH)			Titratable Acidity (Ml. 0.1 N NaOH)	
	Cracker+ Whole Saliva	Cracker+ Sediment		Cracker+ Whole Saliva	Cracker+ Sediment
Day 1	25.1	15.9	Day 4	21.8	8.2
Day 2	19.3	13.1			
Day 3	18.4	11.9	Total acid production	84.6	49.1

The 96-hour adaptation of the periodic neutralization technique produced results which are summarized in Table 2. The figures in this table represent the sum of eight titrations carried out in each 24-hour period. Under these experimental conditions, using salted cracker as substrate, whole saliva is again seen to be more efficient in acid production than sediment alone.

Curves demonstrating the buffering capacities of whole saliva, sediment, and supernatant are shown in Figure 2. Whole saliva and supernatant are shown to have very similar buffering capacities, but salivary sediment has little buffering capacity in either the acid or the alkaline range.

Experimental results of the investigation of the various factors which were considered to play some part in the reduction of acid production by salivary sediment as compared with whole saliva are presented in Table 3 and Figure 3. The addition of amylase to sediment tended to give a slight increase in acid production over that recorded with sediment alone. The addition of buffers in the form of "synthetic saliva," as well as amylase, again gave a slight boost to acid production. However, in neither case did acid production approach that achieved with whole saliva. From the similarity of the diagrams for sediment in saline and sediment in water, we can conclude that

osmotic shock from the suspension of the organisms in water is not an important factor. The very similar diagrams for whole saliva and reconstituted saliva suggest that the process of centrifugation does not physically affect the metabolic activity of the organisms in the salivary sediment.

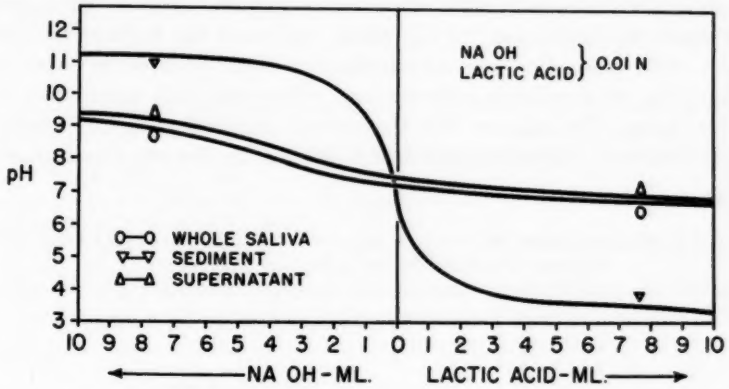


FIG. 2.—Buffering capacities of whole saliva, salivary sediment, and supernatant demonstrated by titration with NaOH and lactic acid.

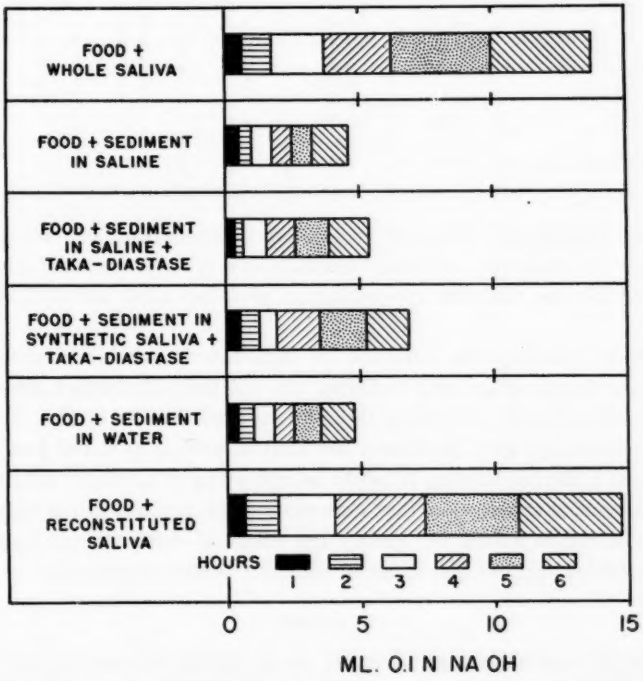


FIG. 3.—Acid production from salted cracker by various salivary fermenting agents. Periodic neutralization technique, with hourly titrations for a total of 6 hours.

Only one of the control tests (food plus amylase) showed any significant change in pH, and this was almost certainly due to bacterial contamination early in the experiment, probably from the electrodes of the pH meter.

DISCUSSION

Our investigations into the relative fermentative capacities of whole saliva, sediment, and supernatant for foods have, on the whole, confirmed the findings reported with pure sugars. Acid production commenced simultaneously with whole saliva and, in reduced amounts, with sediment, whereas acid production with supernatant was delayed several hours. This suggests that the intrinsic glycolytic activity of saliva is located in the sediment (the micro-organisms). Support is also given in this study for

TABLE 3

ACID PRODUCTION FROM SALTED CRACKER BY VARIOUS SALIVARY FERMENTING AGENTS—PERIODIC NEUTRALIZATION TECHNIQUE

TITRATION AT	TITRATABLE ACIDITY (ML. 0.1 N NaOH)					
	Food+ Whole Saliva	Food+ Sediment in Saline	Food+ Sediment in Saline+ Taka-Diastase	Food+ Sediment in Synth. Saliva+ Taka-Diastase	Food+ Sediment in Water	Food+ Recon-stituted Saliva
Hour 1	0.75	0.45	0.4	0.6	0.5	0.7
Hour 2	1.0	0.5	0.2	0.8	0.6	1.2
Hour 3	2.0	0.6	0.9	0.8	0.7	2.0
Hour 4	2.6	0.75	1.1	1.4	0.9	3.4
Hour 5	3.7	1.1	1.2	1.7	0.9	3.6
Hour 6	3.8	1.2	1.6	1.6	1.3	3.9
Total acid production	13.9	4.6	5.4	6.9	4.9	14.8

the concept of Hartles and Wasdell[3] that salivary supernatant contains an adjuvant, in the absence of which the metabolic processes are greatly retarded. These workers[5] also found, as we did, that the reconstitution of saliva after centrifugation restored its activity.

Our efforts to identify the adjuvant in supernatant are inconclusive. We must conclude either that amylase and buffering are not the sole factors involved or that our artificial additions did not adequately imitate these factors as they occur in whole saliva. This experiment may have been too limited, testing as it did just one amylase sample and one buffering system. It might be rewarding if attempts were made to find artificial supplements to sediment that were more representative of the naturally occurring systems in whole saliva by testing the effect of different amylase samples in different concentrations and the effect of different buffering systems.

SUMMARY

The relative fermentative capabilities of whole saliva, salivary sediment, and salivary supernatant upon foods were examined, using the periodic neutralization technique. In no case did sediment or supernatant produce as much acid as did whole

saliva. The intrinsic glycolytic activity of saliva was shown to rest in the sediment, but the supernatant appeared to contain some adjuvant which increased the glycolytic potential of the organisms. In an effort to identify this adjuvant, artificial amylase and buffer systems were added to salivary sediment. While boosting acid production, these artificial additions were not so effective as natural supernatant in restoring sediment to the full glycolytic activity of whole saliva.

REFERENCES

1. Beck, Donald J., and Bibby, Basil G. Acid Production during the Fermentation of Starches by Saliva, *J. D. Res.*, in press.
2. Sreebny, Leo M., Kirch, E. R., and Kesel, R. G. The Location of the Glycolytic Enzymes in Saliva, *J. D. Res.*, **29**:506, 1950.
3. Hartles, R. L., and Wasdell, Marie R. The Metabolism of the Oral Flora. 4. The Invertase Activity of Mixed Human Saliva, *Brit. D. J.*, **97**:231, 1954.
4. Burber, M., Lavine, L. S., Deane, B. C., and Sobel, A. E. Calcification. XIX. Calcification of Transplanted Rachitic Bone, *Proc. Soc. Exptl. Biol. Med.*, **96**:147, 1957.
5. Hartles, R. L., and Wasdell, Marie R. A Comparison of the Metabolic Activity of Salivary Sediment with That of Whole Saliva, *J. D. Res.*, **34**:784, 1955 (Abstr.).

Acid Production during the Fermentation of Starches by Saliva

DONALD J. BECK and BASIL G. BIBBY
Eastman Dental Dispensary, Rochester, New York

It is generally agreed that the bacterial breakdown of carbohydrate-containing foodstuffs into organic acids is of primary importance in the causation of dental caries. It follows that attempts at rating foodstuffs in their importance in caries causation may take into account the speed and amount of acid they produce on fermentation by mouth bacteria.

Although earlier investigators used acid production as a means of comparing foods, there has been only one recent attempt to compare acid production from a large number of foodstuffs. In this Bibby, Goldberg, and Chen[1] mixed 5 cc. of paraffin-stimulated saliva and 5 cc. of 3 per cent food suspension and titrated for total acidity with 0.1 N sodium hydroxide after 4 and 24 hours of incubation at 37.5° C. The range of values obtained for the 105 foods tested was relatively narrow, e.g., at 4 hours, the values ranged from 0.6 to 3.3 cc., with the majority between 1.0 and 2.0 cc.; at 24 hours, the range was 1.0–3.7 cc., with the majority between 1.5 and 3.0 cc.

The technique of Bibby, Goldberg, and Chen can be criticized because it does not allow fermentation to go to completion. During the first few hours of the fermentation process the pH falls to levels of 4 or 5, at which stage further fermentation is inhibited before all the available carbohydrate has been utilized by the bacteria. It seems unlikely that this situation would obtain in the mouth, because the dental plaque, the clearance of saliva and food debris, and dissolution of tooth salts may prevent the existence of low pH conditions for prolonged periods. Thus, theoretically, for as long as food is retained in the mouth, fermentation and acid production may continue. Therefore, it seems that a measurement of acid production from foodstuffs under conditions more closely resembling those prevailing in the mouth would give more meaningful results. This paper reports results obtained with a periodic neutralization technique which provides a continuing environment with a favorable pH for acid production.

MATERIALS AND METHODS

In the periodioc neutralization technique a pH suitable for bacterial fermentation was maintained by repeatedly titrating the test mixtures of food and saliva back to the original pH, using 0.1 N sodium hydroxide and a Beckman pH meter.* The sum of the individual titrations was taken to represent the total acid production.

This investigation was supported in part by a research grant, D-607, from the National Institute of Dental Research, National Institutes of Health, and in part by the American Chicle Company.

Received for publication September 6, 1960.

* Model M with glass electrode No. 43509.

A comparison of the periodic neutralization technique and the single terminal neutralization technique was made by using chocolate cookie, salted cracker, corn flakes, chocolate ice cream, and fresh, ripe banana (30 ml. of 5 per cent food suspension plus 10 ml. of paraffin-stimulated, pooled saliva). One series of samples was titrated to neutrality at hourly intervals for 6 hours, and another series of samples was neutralized only at the end of the 6-hour fermentation. The results of this preliminary investigation indicated that, although hourly neutralization resulted in greater acid production than did terminal neutralization, all the substrate was still not being used. A modified procedure was therefore adopted in which the fermentation was continued for 48 hours, with additional titrations at 12, 24, and 48 hours.

In this modified form, the periodic neutralization technique was employed to investigate the relative acid-forming potentialities of 29 flours. These were as follows: *Wheat*—bleached enriched flour, natural unbleached flour, whole-wheat (graham) flour,* wheat germ. *Corn*—corn starch, yellow corn meal.* *Rice*—extra-long-grain polished rice, natural brown long-grain rice, "Minute" rice, wild rice,* native (Amazon Valley, Brazil) rice. *Rye*—whole rye flour.* *Oats*—oat flour,* "Scotch" oatmeal. *Barley*—medium pearled, large pearled. *Legumes*—green split peas, yellow split peas, baby lima beans,* red kidney beans,* jumbo lentils.* *Tapioca starch*—pearl tapioca, *Farinha seca* (Brazil), *Farinha d'agua* (Brazil). *Edible pastes*—macaroni, spaghetti, egg noodles. *Miscellaneous*—full fat soy flour, potato starch. The controls used were sucrose, glucose, and distilled water.

All test samples were brought to a particle size which would pass U.S. Standard Sieve No. 80 (0.177-mm. mesh) but would be retained by U.S. Standard Sieve No. 200 (0.074-mm. mesh). Where indicated, milling was done in the laboratory with an electric mortar.†

The fermentation mixtures consisted of 1.5 gm. flour suspended in 30 ml. of water (5 per cent suspension) to which was added 5 ml. of paraffin-stimulated saliva pooled from eight individuals. The sucrose and glucose controls consisted of 1.5 gm. of the reagent grade sugars dissolved in 30 ml. of distilled water and in 5 ml. of saliva. The distilled-water control consisted simply of 30 ml. of distilled water and 5 ml. of saliva.

Before fermentation was commenced, the pH values of the flour suspensions and the flour suspensions plus saliva were recorded. In four cases (the two farinha samples and the two barley samples), where the original pH of the flour plus saliva was less than 6.8, the mixtures were adjusted to this arbitrary level of 6.8 with a few drops of sodium hydroxide, and in all subsequent titrations these four mixtures were readjusted to pH 6.8. All other samples produced pH values in the range 6.9–7.8 without further adjustment when the saliva was added to the flour suspension, and, in each case, it was to this original value to which the mixtures were titrated during the course of fermentation.

The samples were incubated at 37° C. in a water bath and shaken vigorously throughout the fermentation period. Titrations with 0.1 N sodium hydroxide were carried out after 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours, using a Beckman pH meter to determine the end point.

* Whole-grain flours. Samples either claimed by the manufacturer on the package to be milled from whole grain or actually milled from whole grain in this laboratory.

† Crescent Heavy Duty Wig-L-Bug No. 6. Crescent Dental Manufacturing Co., Chicago 23, Illinois.

Subjective findings on the degree of odor of putrefaction in the 48-hour cultures, which was found to vary considerably from culture to culture, were recorded.

RESULTS

Acid formation in five food/saliva mixtures, measured by the periodic and terminal neutralization techniques, are presented in Figure 1. A control test of water and saliva showed no appreciable change in pH during the 6-hour fermentation period. All other

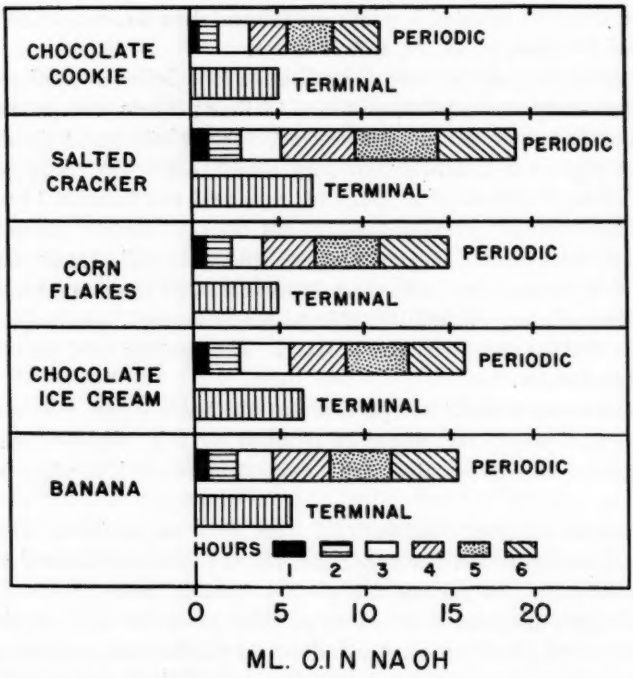


FIG. 1.—Comparison of the periodic neutralization technique (hourly titrations with 0.1 N NaOH) and the terminal neutralization technique (single terminal titration with 0.1 N NaOH) for measuring acid production during 6-hour fermentations of five food/saliva mixtures.

tests showed measurable acid production. Samples subjected to periodic neutralization yielded two to three times as much acid as did those neutralized only at the end of fermentation (Table 1).

The amounts of titratable acid formed by 29 flours and 2 pure sugar controls, fermented with saliva, are shown in Figure 2. The distilled water plus saliva control showed no significant change in pH during the course of fermentation.

DISCUSSION

The most striking findings are the very wide spread in values for acid production from the various test substances and the relatively low acid production from sucrose and glucose.

The acid-production figures for the flours indicate that, in flours derived from any one grain, acid production was greater with the less refined product. This can be seen

TABLE 1

PERIODIC NEUTRALIZATION TECHNIQUE AND TERMINAL NEUTRALIZATION TECHNIQUE FOR MEASURING ACID PRODUCTION DURING FERMENTATION OF FIVE FOOD/SALIVA MIXTURES

PERIODIC NEUTRALIZATION TECHNIQUE	TITRATABLE ACIDITY (ML. 0.1 N NaOH)				
	Chocolate Cookie	Salted Cracker	Corn Flakes	Chocolate Ice Cream	Banana
Titration at:					
Hour 1	0.7	1.0	0.9	0.9	0.9
Hour 2	1.0	1.9	1.4	1.8	1.7
Hour 3	1.8	2.4	1.8	2.9	2.3
Hour 4	2.2	4.3	3.1	3.2	3.1
Hour 5	2.5	4.8	3.9	3.6	3.5
Hour 6	2.7	4.7	3.9	3.4	4.0
Total of six titrations	10.9	19.1	15.0	15.8	15.5
Terminal neutralization technique:					
Titration at hour 6	5.3	7.1	5.0	6.4	5.6

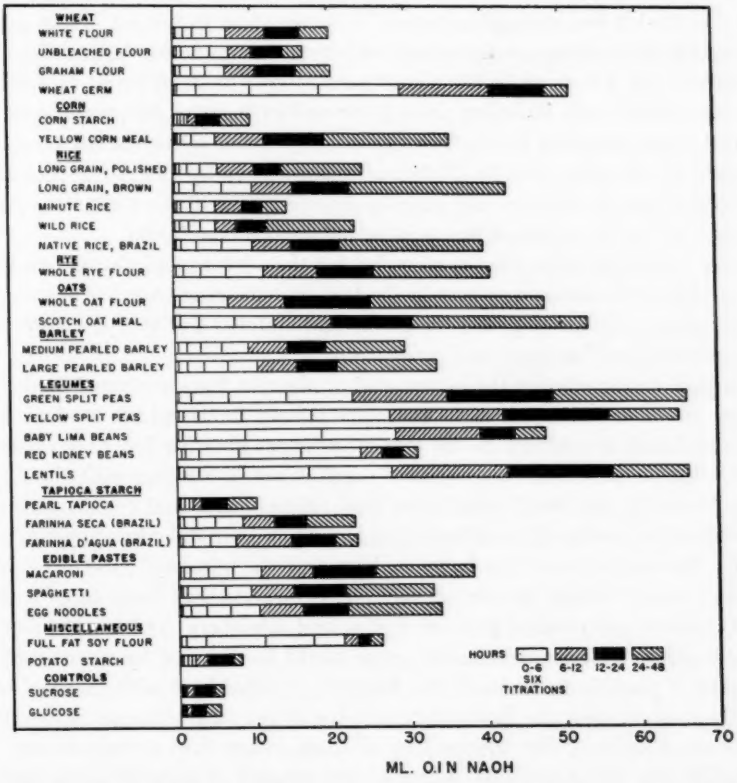


FIG. 2.—Acid production during the fermentation of starchy foods with saliva. Periodic neutralization technique with hourly titrations with 0.1 N NaOH for the first 6 hours and titrations at the end of 12, 24, and 48 hours.

most clearly in the results for the two corn products and for polished rice and natural brown rice. These results suggest that some substance or substances may be present in whole-grain materials that stimulate bacterial metabolism. The similar acid production from white flour and whole-wheat (graham) flour appears to contradict the hypothesis that acid production decreases with increasing refinement. However, since the white flour used was commercially "enriched" by the addition of approximately the same amounts of thiamine, niacin, riboflavin, and iron as is contained in 100 per cent whole wheat, it could be argued that one or all of these four substances could have increased its suitability as a medium for acidogenic organisms.

Low acid production in the later stages of the fermentation of wheat germ, lima beans, red kidney beans, and soy flour suggest that all available carbohydrate was utilized. This theory is substantiated by the very high acid production in the early stages of fermentation, the relatively low initial per cent carbohydrate values for these foods, and the presence of putrefactive odor at 48 hours (suggesting that proteolysis had become the predominant metabolic process). Negative findings for putrefactive odor were recorded in all other tests except yellow split peas, where the odor was doubtful.

The fact that our fermentation mixtures of saliva and farina and barley were sufficiently acid that it was deemed necessary to buffer them to pH 6.8 before incubation, so as to provide a reaction which would not be unfavorable for bacterial growth and fermentation, has a bearing on the significance of acid foods in caries causation. If, in the mouth, acidic foods, including fruit juices and carbonated beverages, must be partially neutralized by saliva before they can undergo active fermentation, it seems likely they would at the same time be diluted or washed away by the saliva, and thus their harmful effect on the teeth would have to depend primarily on their inherent acidity rather than on the formation of new acid by bacterial fermentation.

The low acid-production figures recorded for the sucrose and glucose controls need comment. The explanation may rest in the fact that the reagent grade sugars provided a poorer culture medium than did the grains and starches and that the lower bacterial growth reflected itself in lower acid production.

It is logical to ask whether the higher acid production from unrefined foods indicates that they are more cariogenic than the more refined foods, which produce less acid. Such a conclusion is contrary to the clinical evidence showing low caries incidence in peoples living on unrefined foods (Shaw[2]) and is not in keeping with the findings of Osborn, Noriskin, and Staz,[3] who found that refined sugar and cereal products incubated with saliva produced more tooth decalcification than did corresponding unrefined products. The suggestion of the latter authors that the unrefined products contained a "protective agent" which interfered with decalcification has been supported by the work of Osborn[4] and Jenkins, Forster, Speirs, and Kleinberg[5] and in our laboratories.[6] These indications that enamel decalcification might be modified by agents contained in foods make it possible to reconcile the findings reported here with those of other authors. They also suggest the desirability of using direct measurements of enamel decalcification as an index of the cariogenicity of foods rather than depend on acid production or other less direct methods. Although the amount of decalcification that a foodstuff produces is probably related to the amount of acid produced during fermentation, the relationship does not appear to be a simple one.

SUMMARY

Measurement of acid production by single or repeated neutralization showed much greater acid production in the latter system. The acid production from 29 hours was examined by means of the periodic neutralization technique. Large variations were found between the flours, with acid production generally being greater with whole-grain flours than with the more refined flours. The existence of some "protective factor" in whole-grain flours, reducing their decalcifying potential, was suggested.

REFERENCES

1. Bibby, Basil G., Goldberg, Hyman J. V., and Chen, Eugene. Evaluation of Caries-producing Potentialities of Various Foodstuffs, *J.A.D.A.*, **42**:491, 1951
2. Shaw, James H. Nutrition and Dental Caries. In *A Survey of the Literature of Dental Caries*, pp. 417–507. Publication 225. Washington, D.C.: National Academy of Sciences–National Research Council, 1952.
3. Osborn, T. W. B., Noriskin, J. N., and Staz, J. A Comparison of Crude and Refined Sugar and Cereals in Their Ability To Produce in Vitro Decalcification of Teeth, *J. D. Res*, **16**:165, 1937.
4. Osborn, T. W. B. Further Studies on the in Vitro Decalcification of Teeth, *J. D. Res.*, **20**:59, 1941.
5. Jenkins, G. Neil, Forster, Margaret G., Speirs, R. L., and Kleinberg, I. The Influence of the Refinement of Carbohydrates on Their Cariogenicity, *Brit. D. J.*, **106**:195, 1959.
6. Bibby, B. G., and Soni, N. N. Acid Production and Enamel Decalification in Food-Saliva Mixtures, *J. D. Res.*, **39**:699, 1961.
7. Andlaw, R. G. The Relationship between Acid Production and Enamel Decalcification in Salivary Fermentation of Carbohydrate Foodstuffs, *J. D. Res.*, **39**:1200, 1961.

A Comparison of Two Types of Clinical Examinations: The Effect on Observed Caries Prevalence and Increments

P. R. WEISENSTEIN, JOHN K. PETERSON, and PAUL E. NORRIS

College of Dentistry, Ohio State University, Columbus; North Dakota State Department of Health, Bismark; and Division of Dental Research, Procter & Gamble Company, Cincinnati, Ohio

The influence of the examination method and the precision of the examiner on data collected in clinical examinations are important to all investigators in clinical studies of caries. The Vipeholm Dental Caries Study[1] reported a comparison of examiners after they had been trained to use common diagnostic methods. The precision of the examiners has also been investigated. Slack, Jackson, James, and Lawton[2] compared the results of clinical examinations of four examiners made before and after the establishment of specific diagnostic criteria. A study reported in the International Symposium on Clinical Estimation of Caries Incidence and Increments, September, 1959,[3] showed the effect of seven independent examiners on the observed data in a clinical study; no attempt was made to standardize the analysis of the data obtained by each examiner. In all these studies, considerable variation in diagnosis was observed when different examiners examined the same individuals.

The present study compares data obtained by two examiners who used different methods of clinical examination on the same group of subjects. Examiner A used a slow method of examination for the visual diagnosis of caries. Examiner B used a more rapid method for his clinical examination. Since each examiner was experienced in applying his method of diagnosis, no attempt was made to alter or control the precision of the examiners.

It is well known that caries prevalence as measured by different examiners will vary. The purpose of this study was to evaluate two methods of examination for their influence on the caries *increment*. The data obtained by each examiner were analyzed in the same manner.

EXPERIMENTAL METHODS

The one hundred and eleven Sophomore dental students used as subjects received a dental prophylaxis during the week preceding each examination. Two examinations, a year apart, made on each subject included a clinical examination by each of the two examiners and four bite-wing radiographs (two posterior films on either side of the mouth). Following each examination period the radiographs were read by the examiners, and the observations recorded in red pencil to supplement the visual diagnosis of interproximal lesions in posterior teeth. The time used in the clinical examination was recorded for each examiner. Each examiner used the examination technique he had been accustomed to using for many years, and each used his own instruments.

Examiner A used a conventional dental light, sharpened sickle exploring points, an

Received for publication September 15, 1960.

air blast, and separation in the anterior teeth whenever he felt it was necessary. His average time for the oral visual examination was 5.3 minutes per subject. Examiner B used an intra-oral illuminated mirror and #5 unsharpened explorers. His average time was 1.9 minutes per subject. The same type of examination form was used by both examiners to record the findings. The second examination was recorded without reference to the data of the first examination, in order to obtain independence of observations. The only changes made after all data were recorded were changes involving tooth designation. The necessity for these changes was caused by the early loss of the

TABLE 1

DECAYED, MISSING, OR FILLED TEETH

	Slow Examination			Fast Examination		
	Total	Visual	X-Ray	Total	Visual	X-Ray
DMFT 1958 (av.)	17.95	17.67	0.29	15.79	14.32	1.47
Increments 1958–59 (av.)	0.88	.....	.....	0.16	.....	.....
Standard deviation	1.71	.....	.....	1.32	.....	.....
Error of mean	0.16	.....	.....	0.12	.....	.....
Reversals (av.)	0.27	0.23	0.04	0.56	0.18	0.38

TABLE 2

DECAYED, MISSING, OR FILLED SURFACES

	Slow Examination			Fast Examination		
	Total	Visual	X-Ray	Total	Visual	X-Ray
DMFS 1958 (av.)	42.27	39.48	2.79	36.54	31.82	4.72
Increments 1958–59	3.37	.....	.....	1.62	.....	.....
Standard deviation	3.30	.....	.....	3.11	.....	.....
Error of mean	0.32	.....	.....	0.30	.....	.....
Reversals	1.57	1.13	0.44	2.00	0.68	1.32

first molar, which led to improper designation of the tooth occupying the first molar position. Such error also ocurred when a bicuspid was removed for orthodontic purposes and the designation of the first or second bicuspid differed between examinations. Data were not recorded for the third molars.

RESULTS

The prevalence of decayed, missing, and filled teeth found by each examiner is shown in Table 1. The prevalence of decayed, missing, and filled *surfaces* found by each examiner is shown in Table 2. The frequency distribution of the findings in Table 2 is shown in Table 3. A division of the decayed, missing, and filled surface increments into component parts is shown in Table 4. Table 5 shows the frequency with which diagnoses made in the first examination were reversed in the second examination expressed as a per cent of the total DMF found at the first examination.

DISCUSSION

Arguments have been advanced repeatedly that the criteria for diagnosis and the speed of the examination are not so important if these criteria remain constant through-

TABLE 3

FREQUENCY DISTRIBUTION OF INCREASE IN NEW DMFS DURING 1-YEAR PERIOD

No. of New DMFS	Clinical		X-Ray		Total	
	Fast Exam.	Slow Exam.	Fast Exam.	Slow Exam.	Fast Exam.	Slow Exam.
− 7	0*	0	1	0	0	0
− 6	0	1	1	0	2	0
− 5	0	0	0	1	1	0
− 4	0	0	2	0	4	1
− 3	0	0	5	0	1	1
− 2	4	4	11	2	3	1
− 1	4	4	28	13	12	9
0	27	17	29	46	17	11
1	23	19	14	26	15	11
2	20	15	8	13	16	15
3	14	11	7	7	17	11
4	7	12	2	3	8	13
5	7	11	2	0	5	14
6	3	3	1	0	4	8
7	0	5	0	0	1	6
8	1	4	0	0	2	3
9	0	1	0	0	0	2
10	1	1	0	0	1	0
11	0	1	0	0	1	3
12	0	0	0	0	1	1
13	0	2	0	0	0	0
16	0	0	0	0	0	1

* No. of individuals.

TABLE 4

DMFS INCREMENTS FOR SPECIFIC SURFACES

	Slow Examination	Fast Examination
Total	3.37	1.62
Proximals	1.71	0.63
Occlusals	0.68	0.64
Buccal-linguals	0.98	0.35

TABLE 5

PERCENTAGE OF INITIAL FINDINGS REVERSED

	DMFT			DMFS		
	Total	Visual	X-Ray	Total	Visual	X-Ray
Slow examination	1.5	1.3	13.8	3.7	2.9	15.8
Fast examination	3.5	1.3	25.8	5.5	2.1	28.0

out the length of a clinical field study. If the foregoing were true, we would expect that two examiners would obtain similar increments in a given period of time if they were comparing a group using a caries-preventive agent with a control group. The present study was not designed to test the full concept, since only one group of subjects was used rather than two groups with different treatments.

The data for each method of examination, slow and rapid, were divided into observations made visually and by X-ray. The data in Table 1 indicate that the slower clinical examination procedure yielded a higher total DMF score than did the rapid examination procedure. The fast examiner made up for some of the difference by reference to the radiographs, but he did not reach the mean prevalence for the group found by the slow examiner. The increments for the one-year period, Table 1, shows a fivefold difference in the two examinations (0.88 versus 0.16). With more time for thorough inspection, the slow examiner found increments of 0.88 new DMF teeth and 3.37 new DMF surfaces during the year period. These are $\frac{3}{4}$ tooth and $1\frac{3}{4}$ surfaces per person greater than the rapid examiner. It is of great interest that, despite the difference in findings with each method of examination, the standard deviations were very similar, indicating agreement on the shape of the distribution curve, even though the means were different.

The data in Table 2 show that the slow examination produced a higher total visual DMFS score, as well as a higher increment, than the fast examination. The rapid examiner made up part of the difference when viewing the radiographs.

The data in Table 4 show that the greatest number of lesions was found by both examiners on the interproximal surfaces, and, in keeping with the total DMFS findings by each method, the slow examinations revealed more lesions than the rapid. Speed of examination did not appear to affect occlusal findings in the investigation, but the slower examination revealed more lesions on the smooth surfaces than did the rapid examination.

The problem of reversals in diagnosis has been recognized for many years and was discussed by Radike and Muhler[4] in 1954. Recently, Radike[5] presented a detailed review of a study of the meaning of reversals.

Since we are using the term "reversal" in this report, we would like to discuss it for purposes of clarification. A reversal occurs when an area on a tooth is diagnosed carious on the first examination and non-carious on the second examination. It is probable, if one is using an effective anticariogenic agent or reducing the caries rate by controlled diet on a group of individuals, that there will be a greater number of reversals appearing in the data than if the caries rate is continuously progressing. It also becomes apparent that reversals will occur with any given definition of a carious lesion if the examiner is consistent in his diagnosis and also conducts his second examination without reference to his first examination data. The apparent disappearance of a previously decayed surface has been subtracted from the caries score for each subject during the analysis of the data in this study.

The data in Table 1 show that the slow examiner had a higher total DMF score than the rapid examiner but had a smaller number of total reversals than the rapid examiner. To determine the source of such observations, the reversals in diagnosis were divided into those observed visually and those observed radiographically. The slow examiner had a greater percentage of reversals in the visual examination than did the rapid

examiner when diagnosing carious surfaces (Table 5). This may be due to the greater length of time spent in visual examinations with the resulting greater opportunity for diagnosis at a level where more borderline conditions exist. Finally, the reversals encountered by the slow examiner were very few when observing the radiographs, compared with the rapid examiner, who had to make more diagnosis of borderline conditions when observing the radiographs. A similar pattern occurred in the diagnosis of DMF surfaces, as seen in Table 2. Again, the slow examiner had the greater number of reversals on visual examinations.

The problem of borderline decisions can be better appreciated by referring to discussions of indices used to record caries and their relative merits found in other sources.[6, 7]

The speed of examination alters the findings with respect to caries prevalence and to caries incidence over a 1-year period.

SUMMARY

The purpose of the study was to observe the effect of examination method on a caries increment when the same subjects are examined by two procedures. A group of one hundred and eleven college students was examined by two examiners using different methods of examinations. The slow-examination method showed a mean prevalence of 17.95 DMF teeth at the initial examination, whereas the rapid-examination method indicated a mean of 15.79 DMF teeth. Similarly, the slow-examination method showed 42.27 DMF surfaces per subject, and the rapid-examination method showed 36.54 DMF surfaces per subject.

One year after the initial determination of prevalence, the slow examination showed 0.88 new DMF teeth as a mean, while the rapid examination showed only 0.16 new DMF teeth as a mean. Similarly, the increment for DMF surfaces was 3.37 by the slow method compared with 1.62 by the fast method.

A division of findings into visual and radiographic observations revealed that the slower examiner found most of the diagnosed caries visually and made most of his borderline decisions visually, as expected. The rapid examiner found a smaller number of carious lesions visually but a greater number than the slow examiner when using the radiographs. The rapid examiner made more borderline decisions when examining the radiographs and, therefore, showed more reversals in diagnosis in the X-ray portion of the data.

REFERENCES

1. Quensel, C. E., Gustafsson, B. E., and Grahnen, H. The Vipeholm Dental Caries Study. Reliability of the Method in the Determination of Caries Activity, *Acta Odontol. Scand.*, **11**:365, 1954.
2. Slack, G. L., Jackson, D. L., James, P. M. C., and Lawton, F. E. A Clinical Investigation into the Variability of Dental Caries Diagnosis, *Brit. D. J.*, **104**:339, 1958.
3. International Symposium on Clinical Estimation of Caries Incidence and Increments, Sponsored by the Study Section, National Institute of Dental Research, Ohio State University, September, 1959. The proceedings of this Symposium are to be published in *J. D. Res.*
4. Radike, A. W., and Muhler, J. C. Effect of Reversals on Diagnosis in the Interpretation of Clinical Caries Tests (abstr.), *J. D. Res.*, **33**:682, 1954.
5. Radike, A. W. A Study of the Meaning of Reversals. Essay presented at the International Symposium on Clinical Estimation of Caries Incidence and Increments (see Ref. 3).
6. Russell, A. L. An Appraisal of the Value of Indices Proposed as Epidemiologic Aids in the Practice of Dental Public Health, *Proceedings of the Fourth Workshop on Dental Public Health, April, 1956*, p. 63. School of Public Health, University of Michigan, Continued Education Series #67.
7. *Report of a Conference To Develop Uniform Standards and Procedures* in *Clinical Studies of Dental Caries*, pp. 8–21. Chicago: American Dental Association, 1955.

Evaluation of Techniques for Analyzing Cavity Design for Amalgam Restoration

DAVID B. MAHLER, LOUIS G. TERKLA, and LEONARD N. JOHNSON
University of Oregon Dental School, Portland, Oregon

The ability of an amalgam restoration to withstand the forces of mastication depends on two factors: (1) the strength of the amalgam, which realizes its full potential by proper manipulative technique, and (2) the distribution and magnitude of the internal stress, which is determined by the nature of force application and the design of the restoration.* While manipulative technique and its effect on the strength of restorative materials has undergone extensive investigation, the evaluation of the design of the restoration has received little emphasis. Present concepts of design have obtained most of their support from clinical observation of success and failure. As a consequence, substantiation of these concepts with quantitative techniques of evaluation is indicated.

Before this evaluation can be accomplished, an appropriate technique of design analysis must be selected and validated. It is the purpose of this paper to report on the evaluation of several of these techniques.

MATERIALS AND METHODS

Techniques evaluated.—In general, the techniques evaluated consisted of models of prepared teeth in which restorations were placed and then fractured by the application of forces similar in nature to those present during mastication. A comparison of the fracture loads was then used as a means of evaluating the efficacy of the restoration design.

The most straightforward method for design analysis would be to restore natural teeth with amalgam and subject these restored teeth to forces similar in nature and magnitude to those present during mastication. It might be anticipated that the precision of this technique would be affected by morphologic differences between natural teeth, inexact duplication of a given cavity design in the various test teeth, and variation in the strength of the condensed amalgam. Despite these objections, tests on natural teeth were conducted to determine whether the results of laboratory-designed methods of evaluation would correlate with those derived from these approximated clinical conditions. Two laboratory-designed tests were selected for evaluation.

The first test consisted of duplicating a prepared natural tooth in metal and condensing amalgam into this metal model. A method similar to this was initially reported

This investigation was supported by U.S.P.H.S. research grant D-514 from the National Institute of Dental Research, National Institutes of Health.

Received for publication September 27, 1960.

* The design of the restoration is defined by the complete morphology, both external and internal. This would be determined by the cavity preparation and the occlusal and proximal carving.

by Lampshire.[1] In this technique the variability of tooth and cavity form was eliminated, but the factor of strength variation of the amalgam was retained. A comparison of the results of this technique with the natural tooth-amalgam results would also indicate the influence of the properties of the supportive structure on the resistance of the amalgam restoration.

The second test was selected from engineering practice and is known as the "brittle-model" or "plaster-model" method. In this technique the variability of tooth and cavity form, as well as the variability of the strength of amalgam, was eliminated. Justification for the choice of this method was based on several factors. First, amalgam behaves like a brittle material under the rapid rates of load application present during mastication, which should correlate with the fracture characteristics of plaster. Second, the models are easily prepared and the morphology exactly reproduced. Third, the method is three-dimensional in nature and does not require extrapolation, as needed by other methods—for example, the two-dimensional technique of photoelasticity.

Method of test.—Four different cavity designs of a disto-occlusal preparation in an upper first bicuspid were selected. These consisted of the following:

1. Design A was prepared with no retentive features but employed presently accepted principles of cavity design. The occlusal surface of the restoration was carved slightly to produce a flat plane because of the difficulty in duplicating exactly the original morphology.

2. Design B was design A altered in the following manner. The bucco-occlusal and linguo-occlusal walls were undercut, the axiopulpal line angle was rounded, retention grooves were placed with a gingival margin trimmer in the axiobuccal and axiolingual line angles of the proximal box, and the gingival wall was sloped to meet the axial wall at less than a 90° angle.

3. Design C was the same as design A except that the pulpal wall was deepened, as advocated by Lampshire.[1] The pulpal wall was rounded, starting at two-thirds the distance of the mesiodistal dimension of the occlusal wall and tapered downward toward the axiopulpal line angle. At this point the drop in elevation was estimated to be $\frac{3}{4}$ mm.

4. Design D was the same as design B except that the proximal retention grooves were cut with a No. 700 tapered fissure bur instead of a gingival margin trimmer.

All these designs were placed in natural teeth and restored with amalgam, in technic metal teeth and restored with amalgam, and in densite teeth which were restored with plaster. The specific details of these techniques follow.

Amalgam in natural teeth.—Cavity preparations were made in 80 extracted upper first bicuspids, each of the four preparations being duplicated 20 times. The teeth were mounted in rings filled with hydrocal and oriented at 6° from the long axis, so that the load would be applied against the marginal ridge. The load was applied through a $\frac{1}{16}$-inch steel ball to approximate a deleterious loading condition in the mouth. A matrix band was placed and secured with modeling composition, after which the amalgam was manipulated and inserted by means of acceptable clinical technique. In order to achieve a more consistent residual mercury content, a 1:1 proportioning of mercury and amalgam alloy* was used, and no mercury was removed during condensation. The amalgam

* S. S. White New True Dentalloy.

was carved to produce a flat occlusal surface and a proximal surface contoured to reproduce the original tooth morphology. In Figure 1 is shown a schematic sketch of a mesiodistal cross-section through the tooth and restoration, indicating the direction of the load application. This same position and direction of load application were duplicated in all subsequent tests. One week after packing, the samples were tested, and the loads to produce fracture were recorded.

Amalgam in technic metal teeth.—A natural tooth was selected which was considered a representative upper first bicuspid (typical morphology and dimensions corresponding to Black's measurements[2]). Cavity design A was cut, and an impression of

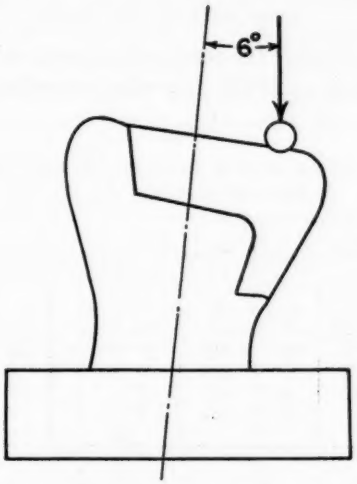


FIG. 1.—Schematic sketch of mesiodistal cross-section of tooth, indicating the nature of load application.

this prepared tooth was made in a cold molding rubber.* Wax patterns were made from this mold and were reproduced in metal† by the dental casting procedure. The cavity design in this same tooth was then altered and reproduced in a similar fashion to produce castings of teeth with cavity designs B and D. Cavity design C was produced by cutting the metal teeth of design A. In this manner, the morphology of tooth and cavity form were exactly reproduced for each specific design variation. Twenty test models were prepared for each design. Amalgam restorations were placed in these metal teeth, and the same conditions of test were followed as specified for the natural teeth.

Plaster-model technique.—An upper first bicuspid (8 × actual size) was carved in wax and duplicated in plaster, using a cold molding rubber mold. Cavities, according to the designs to be tested, were prepared in these plaster models, and these, in turn, were duplicated in a dental gypsum material (densite). A matrix of self-curing acrylic resin was made to cover completely the occlusal and distal contours of the tooth. Model plaster‡ was poured into the cavity preparation, and the matrix was kept in position

* Gra-Tuffy, Permaflex Mold Co.

† Spyco Technic Metal.

‡ Ransom and Randolph Co.

until the plaster hardened. Prior to pouring the plaster, the improved stone teeth were painted with several coats of butyrate model dope* to prevent adhesion between the tooth model and the plaster restoration. The plaster was mixed at a water/powder ratio of 0.70 and stored for 2 weeks at laboratory conditions (75° ± 5° F., 50 ± 10 per cent relative humidity). The models were then tested. The rate of loading was selected at 0.5 inch per minute to insure a straight stress-strain relationship to the breaking point (a requirement of the plaster-model technique). The load was applied at the same position and at the same angulation as for the amalgam tests. Since the plaster models were 8 × actual size, the loading ball diameter was increased from $\frac{1}{16}$ to $\frac{1}{2}$ inch.

RESULTS AND DISCUSSION

In order to determine whether the structural behavior of a given restorative design can be predicted under oral conditions, a specific evaluating technique should be capable of (1) demonstrating a reasonably small variation of the test data, to distinguish small but clinically significant differences in design; (2) predicting the magnitude of the force to cause fracture (desirable but not essential); (3) predicting whether a difference exists between any two designs; (4) predicting which design is better; and (5) predicting how much a given design is better than another. The data derived from this investigation have been presented in a manner which will demonstrate whether the techniques studied satisfy the above requirements.

TABLE 1

LOADS PRODUCING FRACTURE OF RESTORATION FOR VARIOUS TECHNIQUES OF EVALUATING STRUCTURE DESIGN

Design	Natural Tooth with Amalgam				Metal Tooth with Amalgam				Densite Tooth with Plaster			
	$\bar{X}$*	σ†	$\sigma_{\bar{X}}$‡	V_σ§	$\bar{X}$	σ	$\sigma_{\bar{X}}$	V_σ	$\bar{X}$	σ	$\sigma_{\bar{X}}$	V_σ
A.....	77	14	6	17	103	10	5	10	178	24	10	13
B.....	96	18	8	18	108	9	3	8	191	20	9	10
C.....	108	26	12	24	111	9	5	9	199	24	10	12
D.....	123	20	10	16	126	9	6	9	302	23	10	8

* Arithmetic mean in pounds.
† Standard deviation in pounds.
‡ Twice the standard error of the mean in pounds (95 per cent confidence).
§ Coefficient of variation in per cent.

In Table 1, the arithmetic mean, standard deviation, standard error of the mean, and coefficient of variation are indicated for the 20 fracture loads recorded for each design, using the three analyzing techniques studied.

The coefficient of variation,† a measure of variability, was extremely high for the natural tooth-amalgam tests, as was anticipated, but was within reasonable limits for the metal tooth-amalgam and plaster tests. This result demonstrates the effect of the varying morphology of natural teeth on the strength of the restoration and reflects the difficulty in using this technique for evaluating restorative design. It might be antici-

* Testors Corporation.

† Coefficient of variation = standard deviation/arithmetic mean.

pated that minor differences in design could not be distinguished by this technique. In addition, since there was no morphologic variation in either the metal tooth-amalgam tests or plaster tests, the variability of test results must be due to the inherent variability in the strength of these materials, and, since the coefficient of variation was approximately the same for these two tests, the inherent strength variation of amalgam must be similar to that of plaster.

The fact that no statistical difference existed between the strength of the restoration for the natural tooth-amalgam tests and for the metal tooth-amalgam tests (designs C and D) indicates the lesser role played by the mechanical properties of tooth structure in supporting the restoration. The higher values recorded for the metal tooth tests versus the natural tooth tests for designs A and B were considered to be caused by the support offered by the surface roughness of the technic metal castings and/or adhesion of the amalgam to the metal. This support would be more pronounced in the designs exhibiting lower resistance to fracture, and it points to the advisability of investigating other model materials for this purpose.

TABLE 2

DETERMINATION OF WHETHER STATISTICAL DIFFERENCE EXISTS BETWEEN CAVITY DESIGNS

Designs Compared	Natural Tooth with Amalgam	Metal Tooth with Amalgam	Densite Tooth with Plaster
A versus B.....	Yes	No	Yes
A versus C.....	Yes	Yes	Yes
B versus D.....	Yes	Yes	Yes
A versus D.....	Yes	Yes	Yes
B versus C.....	No	No	No
C versus D.....	Yes	Yes	Yes

In Table 2 are shown the results of a statistical comparison of the means using the *t*-test at the 95 per cent confidence level. The designation of "No" indicates that 95 per cent of the time the means compared belong to the same universe, whereas "Yes" indicates that the means are significantly different. The predictability of a difference in design by a given model technique would be reflected in whether or not a statistical difference could be demonstrated when compared with that for natural teeth with amalgam. In the six comparisons evaluated, only one lack of correlation appeared, and this was in the metal tooth-amalgam test for design A versus B. Once again, the reason for this was thought to be some adhesion between the amalgam and the cast metal surface. Tests are presently being conducted with other model materials which would not exhibit this adhesion.

The plaster-model method demonstrated a 1:1 correlation for all design comparisons and is therefore a valid technique for predicting whether or not a difference exists between the resistance to fracture of various designs of amalgam restorations.

In Figure 2, a bar graph is used to represent the data which are given in Table 1. The heights of the bars are the arithmetic means of 20 test runs for each condition, and the lengths of the small bars represent twice the standard error of the mean at the 95 per cent level of confidence. From this graph, the amount of difference between the various

designs is illustrated, as well as which designs offer more resistance to fracture. In going from design A through D, all the evaluating techniques demonstrate increases in strength in a stepwise fashion. However, the metal tooth-amalgam tests appear insensitive for designs A and B when compared with the natural tooth-amalgam tests, again reflecting the possibility of adhesion between amalgam and the metal castings. The plaster tests show a proportionally larger increase for design D than demonstrated by the natural tooth-amalgam tests, which introduces some doubt as to the ability of the plaster method to predict the amount of benefit derived from a particular design variation.

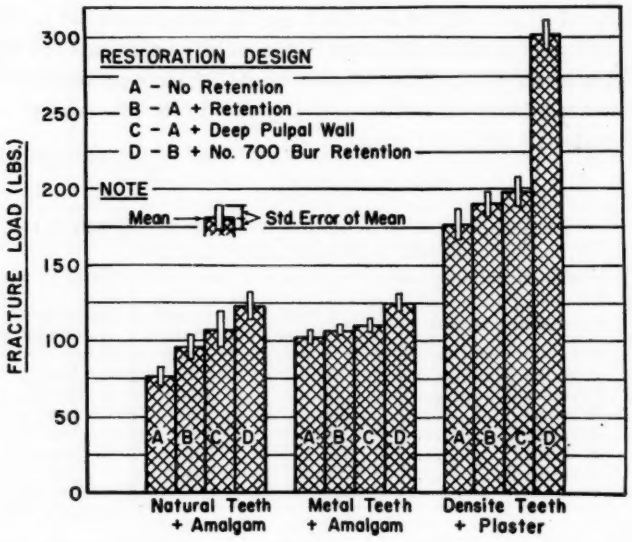


FIG. 2.—Fracture resistance of class II amalgam-type restorations, as determined by various techniques of structural analysis.

Although the primary purpose of this phase of the project was to determine the validity of methods for evaluating restorative design, some information on design can be derived. In Figure 2, it appears that the combined features of undercut buccal and lingual occlusal walls, a rounded axiopulpal line angle, a sloping pulpal wall, and proximal retention grooves—design B—increase the strength of the restoration slightly over design A, which has no retention. Design C, which increases the depth of the pulpal wall and has no retention, is not statistically different from design B with retention. Design D, which is design B with proximal retention cut with a No. 700 bur rather than with a gingival margin trimmer, demonstrates the greatest increase in strength. These results are preliminary, since only one type of loading—static loading to fracture—was utilized. It is planned to investigate two other types of loading which are characteristic of oral conditions, namely, a constantly applied load (flow) and alternate loading (fatigue). When the results of these latter tests are derived, the analysis of design variations will be more meaningful.

SUMMARY AND CONCLUSIONS

The purpose of this report was to evaluate several techniques for the analysis of amalgam restorative design. Amalgam was condensed into prepared cavities of natural teeth and technic metal teeth, and plaster was poured into prepared cavities of densite teeth (plaster-model method). These restorations were subjected to simulated masticatory forces, and the loads to cause fracture were recorded. The following results were obtained:

Natural tooth-amalgam tests demonstrated a greater variability than would be considered desirable from a design analysis standpoint.

Metal tooth-amalgam tests demonstrated insensitivity to some design variations, possibly because of surface adhesion between the amalgam and the metal tooth.

Plaster tests indicated greater benefits from some design variations than was demonstrated by amalgam in natural teeth.

Both metal tooth-amalgam tests (with one exception) and plaster tests could predict whether differences in designs existed and whether a particular design was better.

In conclusion it appears that the most promising evaluating technique would be one using actual-size models of natural teeth into which amalgam could be condensed. The use of models insures reproducibility of morphology, but care must be taken to insure that the model material offers the same support to the amalgam as offered by natural teeth.

REFERENCES

1. Lampshire, Earl L. The Relation of Cavity Form to Failure of Compound Silver Amalgam Restorations in Primary Molar Teeth. Thesis, University of Nebraska Dental School, 1950.
2. Black, G. V. *Descriptive Anatomy of the Human Teeth*, p. 16. 4th ed. Philadelphia: S. S. White Dental Manufacturing Co., 1902.

Further Studies on the Presence of Se^{75} in Rat Saliva and Teeth after Intracardiac Injection of Radioactive Sodium Selenite

C. K. CLAYCOMB, F. M. SORENSON, D. C. GATEWOOD, E. B. JUMP, and M. E. WEAVER

Departments of Biochemistry and Anatomy, University of Oregon Dental School, Portland

The possible relationship between high dietary selenium intake and various disease conditions of animals and man has been suggested in accounts by Smith and Westfall,[1] by Hadjimarkos, Storvick, Remmert, and Bonhorst,[2, 3] and by Tank and Storvick[4] which postulate a relationship between dental caries and high selenium intake. Hadjimarkos and Bonhorst[5] have reported finding selenium in human teeth. Experiments in our laboratory using a radioactive isotope of selenium administered parenterally to rats have shown that this selenium enters the teeth of these animals subsequent to its administration. We were also able to demonstrate a rapid uptake of selenium by human and rat teeth which were imbedded in a variety of solutions of Se^{75} from radioactive rat feces and from saliva made radioactive by addition of small quantities of Se^{75}. These findings suggested the possibility that selenium might enter the teeth of animals and man not only via blood circulation but also from oral fluids which constantly bathe the teeth.

Experiments in which we administered Se^{75} as selenite to adult male rats have revealed that the radioactive selenium could be detected in the saliva of these animals as early as 5 minutes post-injection and up to at least 24 hours post-injection. This was found to be true for non-stimulated saliva as well as for pilocarpine-stimulated saliva.

Recently we reported that relatively large quantities of active selenium were present in all the major salivary glands within minutes after isotope injection.[6] Our data at that time indicated that at least one of these glands might be concentrating the selenium from the blood. The question of the mechanism of selenium transfer to the saliva thus arose.

The purpose of this paper is to report the results of similar, but more conclusive, studies involving the uptake and distribution of selenium in teeth, saliva, and salivary glands of the rat following intracardiac administration of Se^{75}.

MATERIALS AND METHODS

Animals.—Male Sprague-Dawley rats were used throughout these experiments. All animals were kept in our laboratory for at least 5 days prior to their use. Daily weight

This study was supported by U.S.P.H.S. research grants D-739 and D-860 from the National Institute of Dental Research, National Institutes of Health.

Received for publication October 1, 1960.

records were kept for each animal, so that normal weight gain could be established before their use as experimental animals. Body weights of the rats were between 230 and 300 gm. at the time they were placed in experimental groups.

Stock injection solution.—The isotope used throughout these experiments was in the form of selenous acid.* From this a stock injection solution was prepared, using the selenous acid and 0.072 M phosphate buffer. The pH was adjusted to pH 6.8, and the final phosphate concentration was 0.067 M. Final Se concentration of the stock injection solution was 160 μg/0.1 ml. The total radioactivity was 28.3 μc/0.1 ml.

A single intracardiac injection of 0.1 ml. of this stock injection solution was the only dose used for the animals comprising this study.

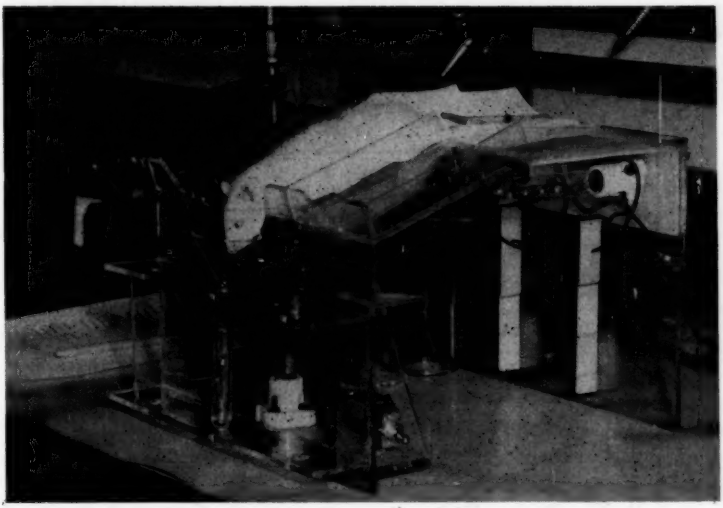


FIG. 1.—Plastic rack for collecting pilocarpine-stimulated saliva from nembutal-anesthetized rats, modified from Bernarde, Fabian, Cosen, Hoppert, and Hunt.

Anesthesia.—All animals were ether-anesthetized prior to intracardiac injection of the isotopes, and nembutal anesthesia (3 mg/100 gm body weight intraperitoneally) was used prior to pilocarpine administration and saliva collection procedures.

Saliva collection.—Following nembutal anesthesia, pilocarpine nitrate (5 mg/kg body wt.) was administered subcutaneously, and the animal was immediately placed on a saliva collection rack modified from Bernarde, Fabian, Cosen, Hoppert, and Hunt[7] (Fig. 1). Saliva was collected for 5 minutes measured from time of pilocarpine injection. Total saliva volume was then determined, and a unit volume removed for counting.

Sacrifice and tissue removal.—When saliva collection was complete, the anesthetized animal was sacrificed by producing a surgical pneumothorax. The heart was immediately exposed, and blood samples were drawn by means of a heparinized syringe and needle. Appropriate portions of the blood were then used for counting procedures and for hematocrit determinations. Following blood withdrawal, the major salivary glands

* Obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

were quickly excised, and approximately equal samples of each were placed in previously weighed gelatin capsules. The tissues were immediately weighed and counted. After gland removal, the lower jaw of each animal was removed and placed in an appropriately labeled container. The lower incisors were later removed, cleaned, weighed, and the radioactivity determined. The entire procedure was so organized that, for any given tissue except teeth, the time from beginning of collection to completion of weighing and counting was never greater than 10 minutes. The activity of all teeth was counted at the end of each day's run. The rapidity with which the tissues were handled and the added protection offered by the gelatin capsules reduced errors in weighing due to water loss to a minimum.

Counting procedures.—All counting was done in a deep-well scintillation counter.*

*Tracerlab Model P20CW with Thallium-activated Sodium Iodide well crystal and Model "1000" scaler.

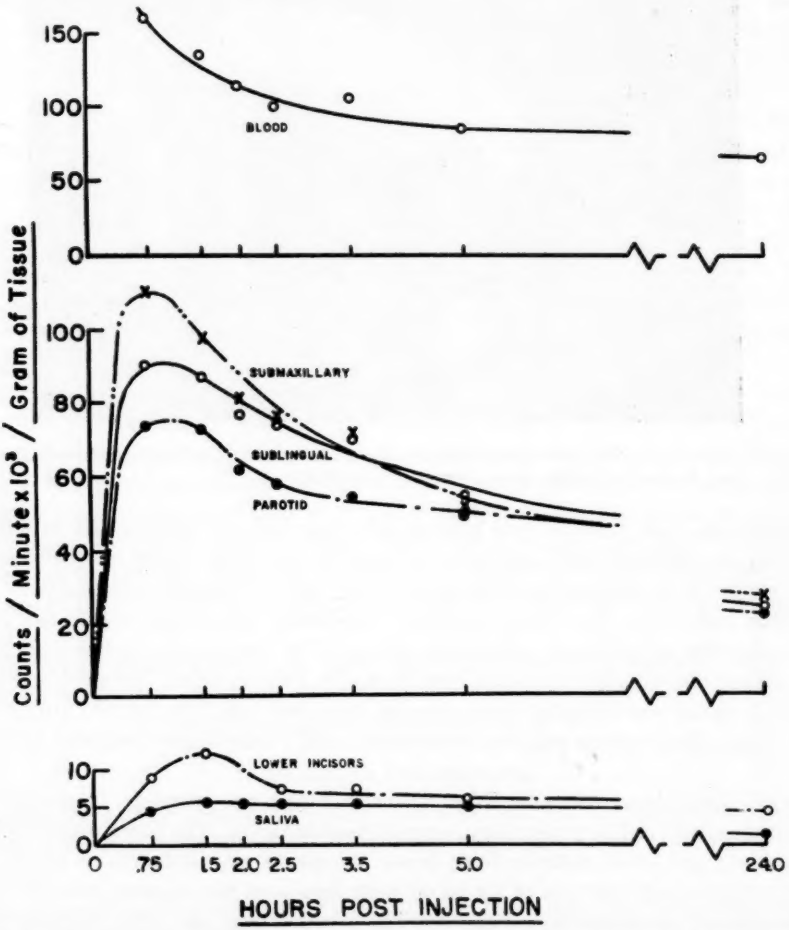


FIG. 2.—Tissue activity relative to blood activity at indicated times post-isotope injection. The percentage of injected activity remaining in the blood at these intervals is shown by the upper graph.

Counting efficiency for the isotope used in this study was approximately 40 per cent. Background and standard counts were made periodically throughout each daily run, to assure proper functioning of the counting equipment. Throughout all experiments, a special effort was made to count samples of similar volume, in order to minimize errors of counting and of weight conversion factors when calculating activity per unit weight.

Experimental design.—The time intervals to be studied and the numbers of animals to be included in each group of our study were selected on the basis of our previous findings; i.e., for those time periods between 1.5 and 5.0 hours, ten animals were used in each group because our earlier results indicated that this would be the critical time interval in our study. All other time periods contained five animals per group. Because it was impossible to complete more than five or six animals on any given day, we felt

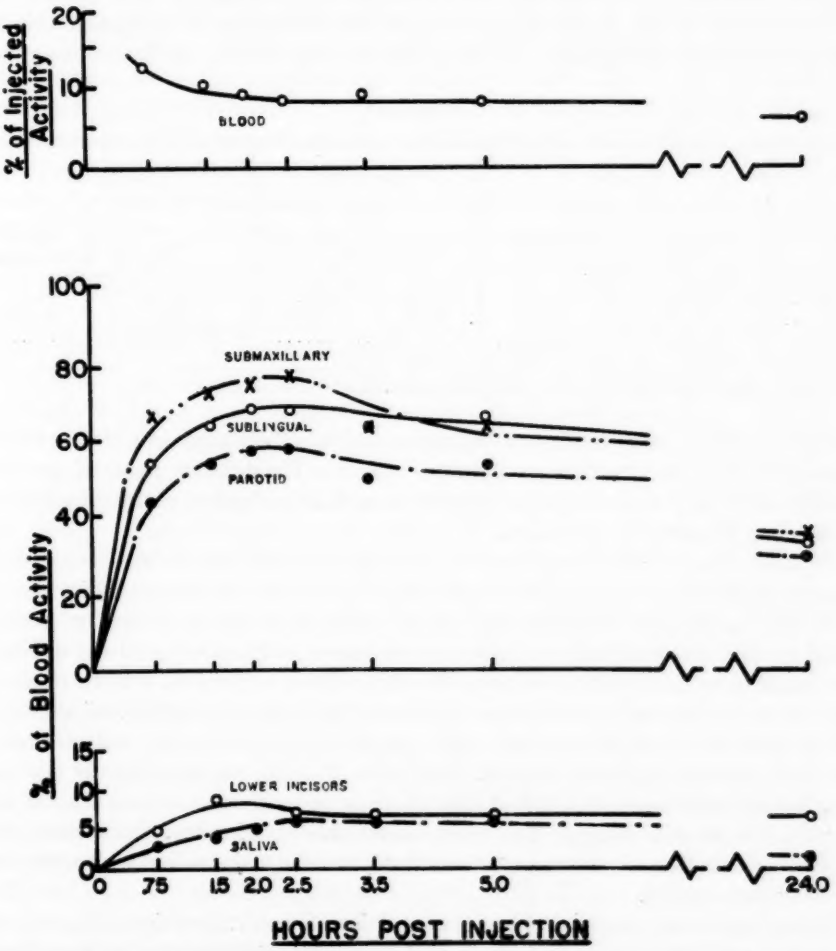


FIG. 3.—The average amount of radioactivity present in tissues assayed at indicated times of sacrifice after a single intracardiac injection of $NA_2 SE^{75} O_3$.

that our best approach would be to do a single five-animal group per day. To avoid mixups and to facilitate time organization, all five animals used on any given day were from the same time group, but the order of groups was randomized, and we had duplicate groups for those time periods which proved to be most critical to this study. In this way we were able to determine that our techniques did not produce significant variation from day to day.

RESULTS

As in our previously reported study, we found that the greatest concentration of Se^{75} in the salivary glands occurred at the earliest time period studied (45 minutes post-injection) and that the amount of Se^{75} in these tissues continued to decrease in all subsequent time intervals up to 24 hours post-injection (Figs. 2 and 3). These data confirm our previous finding that the salivary glands are permeable to selenium but suggest that these glands do not concentrate the selenium prior to its passage into the saliva. It was interesting that the curve representing activity in the teeth of these

TABLE 1*

Group	Parotid		Submaxillary		Sublingual	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
A.....	64.8	±12.0	79.5	±15.5	76.1	±12.3
B.....	68.7	±11.8	79.6	±13.3	79.0	± 6.6
P values	$0.70>P>0.50$	.....	$P>0.90$	.....	$0.70>P>0.50$	.....

* Values reported as per cent of the blood activity. Group A, pilocarpine; Group B, saline.

animals seemed to parallel those of the salivary glands. This suggests a similar mechanism of entry of Se^{75} in all these structures (Fig. 3). The different shape of the saliva activity curve may indicate that a different or additional mechanism may be involved in the entry of selenium into saliva.

Because all rats in this study received pilocarpine immediately prior to removal and counting of the glands, we felt that it was important to rule out the possibility that the sialogogue caused the glands to expel stored selenium, resulting in lowered salivary gland activity measurements and, thereby, erroneous conclusions as to the ability of the glands to concentrate the selenium. For this purpose we selected ten rats of similar age, sex, and weight and divided them into two equal groups (A and B). All animals of both groups were anesthetized with ether and injected (intracardiac) with 0.1 ml. of our stock selenium injection solution. Two hours after isotope injection, the rats were anesthetized with nembutal, and all animals of group A given our usual dose of pilocarpine. The animals of group B received similar volumes of saline. Ten minutes after pilocarpine injection, all animals were sacrificed, and the major salivary glands of each removed for counting.

Table 1 shows the mean values and standard deviations obtained for each group and tissue type. Group comparison tests showed no significant differences between tissues of the two groups (Table 1). It was therefore concluded that pilocarpine as used in

these and previous studies had no significant effect upon the amounts of selenium present in the salivary glands. These findings lend further support to our belief that, under the conditions employed, the salivary glands do not concentrate selenium.

DISCUSSION

It has been reported by McConnell that selenium, administered to dogs, is quite rapidly bound to (or incorporated in) many of the plasma and cellular proteins of the blood.[8, 9] Work in our laboratory confirms these findings. We have been able to demonstrate a very rapid association of Se^{75} with plasma and cellular constituents in the blood of rats following intracardiac administration in the form of selenite. As early as 10 minutes post-injection, approximately 70 per cent of the total activity in the blood is closely associated with the cellular elements and the acetone-precipitable fraction of plasma; and even larger portions of the total blood activity are in some way bound to these constituents as time progresses. Based upon our results and upon the work of others, we suggest the following as a possible general explanation of the events which follow intracardiac administration of Se^{75} in rats.

At the moment after injection, a small portion of the plasma contains all the injected selenium in the form of selenite. Very rapidly this small portion of plasma is diluted by the circulating intravascular blood. At the same time, more and more of the selenium becomes intimately associated with many of the constituents of the plasma (probably to a great extent with the various plasma proteins). The mechanism of association with these components is not known at present, though the work of several investigators suggests that at least some of the Se^{75} may replace sulfur in the amino acids of several plasma proteins.

Since it is known that a considerable amount of the plasma proteins in the animal always remains in the extravascular spaces and since an intravascular-extravascular circulation of plasma in the organs is constantly maintained in healthy animals, it is not surprising that, within minutes after injecting the isotope into the blood, sizable amounts of isotope are detected in almost any tissue of the body. Our data show that during the first hour post-injection the measured activity per unit weight of blood drops very rapidly to approximately 12 per cent of the calculated initial activity (Fig. 2). Although we recognize that a small portion of the injected selenium is excreted via various routes during this time, most of the early loss of activity from the blood must logically be ascribed to its distribution to the exravascular spaces of the body. The relatively free interchange of intra- and extravascular materials within most organs and tissues has been well established. This being the case, it is logical to assume that tissues which have free circulation of blood throughout and which do not actively remove or concentrate the radioactive element should reflect the concentrations of isotope in the "freely diffusable" portion of the blood which supplies them. Also it is to be expected that the relative amount of activity per unit weight of these tissues should reflect, generally, the relative volume of blood circulating through them per unit time. Inspection of the curves presented in Figures 2 and 3 reveals that just such a situation may exist in the three salivary glands and the teeth of rats. Note that these three organs all present the same pattern and that this pattern reflects the concentration of isotope in the blood which had been flowing through them just prior to their removal for study. Note also that the position of each curve indicates, in a general fashion, the relative blood

supply to each organ. Submaxillary and sublingual are highest, parotid gland is significantly lower at all points along the curve, and the teeth take a still lower position.

The curve which depicts activity per unit weight of saliva is interesting in that it does not seem to reflect the blood selenium concentrations as do the other organs studied. This non-conformity may indicate a different or additional mechanism which helps to determine or regulate the amount of selenium which reaches the saliva from the blood.

SUMMARY

Results of a study of the uptake of selenium^{75} by salivary glands, teeth, and saliva of the rat following intracardiac administration are presented. The following conclusions were drawn: The salivary glands of the rat are permeable to selenium. The salivary glands apparently do not store or concentrate Se^{75} during the first 24 hours after administration. Selenium can be found in the extravascular portions of the teeth of rats within a short time of isotope administration. The amount of Se^{75} which will be found in the salivary glands and teeth at any given time post-administration depends primarily upon the concentration of the isotope in the "freely diffusable fraction" of the blood and upon the volume of blood flow through the organ per unit time.

The authors gratefully acknowledge the assistance of Mrs. Ethel Dvorak.

REFERENCES

1. Smith, M. I., and Westfall, B. B. Further Field Studies on the Selenium Problem in Relation to Public Health, *Public Health Repts.*, **62**:1375, 1937.
2. Hadjimarkos, D. M., Storvick, C. A., and Remmert, L. F. Selenium and Dental Caries: An Investigation among School Children of Oregon, *J. Pediat.* (St. Louis), **40**:451, 1952.
3. Hadjimarkos, D. M., and Bonhorst, W. The Trace Element Selenium and Its Influence on Dental Caries Susceptibility, *J. Pediat.* **52**:274, 1958.
4. Tank, G., and Storvick, C. A. Effect of Naturally Occurring Selenium and Vanadium on Dental Caries, *J. D. Res.*, **39**:473, 1960.
5. Hadjimarkos, D. M., and Bonhorst, C. W. The Selenium Content of Human Teeth, *Oral Surg., Oral Med., Oral Path.*, **12**:113, 1959.
6. Claycomb, C. K., Gatewood, D. C., Sorenson, F. M., and Jump, E. B. Presence of Se^{75} in Rat Saliva after Intracardiac Injection of Radioactive Sodium Selenite, *J. D. Res.*, **39**:1264, 1960.
7. Bernarde, F. W., Fabian, F. W., Cosen, S., Hoppert, C. A., and Hunt, H. R. A Method for the Collection of Large Quantities of Rat Saliva, *J. D. Res.*, **35**:326, 1956.
8. McConnell, K. P., and Cooper, B. J. Distribution of Selenium in Serum Proteins and Red Blood Cells after Subcutaneous Injection of Sodium Selenate Containing Radioselenium, *J. Biol. Chem.*, **183**:459, 1950.
9. McConnell, K. P., Wabnitz, C. H., and Roth, D. M. Time Distribution Studies of Selenium-75 in Dog Serum Proteins, *Texas Repts. Biol. and Med.*, **18**:438, 1960.

The Influence of Antibiotics on the Periodontal Syndrome in the Rice Rat

*JAMES H. SHAW, DERRICK GRIFFITHS, and AINA M. AUSKAPS**

Harvard School of Dental Medicine, Boston, Massachusetts

The administration of penicillin or streptomycin in the diet of rice rats has been shown to result in major reductions in the periodontal syndrome.[1] The lesions of the soft tissues were benefited particularly by this procedure, while the lesions of the calcified tissues were influenced much less dramatically. In an effort to understand more fully the role of the antibiotics in this syndrome, polymyxin B sulfate,† erythromycin glucoheptonate,‡ and a benzylpenicillin preparation§ have been evaluated at various levels in the diet on a preventive basis, while a benzylpenicillin preparation, streptomycin sulfate|| and oxytetracycline# hydrochloride have been tested for their curative ability.

EXPERIMENTAL METHODS

The first experiment was conducted with rice rats raised in our colony to test the influence of dietary supplements of polymyxin B sulfate and erythromycin glucoheptonate on the incidence of the periodontal syndrome. The selection of polymyxin B sulfate and erythromycin glucoheptonate as suitable antibiotics to test in this experiment was made on the basis of a commonly used type of in vitro test. Standardized filter-paper disks impregnated with known amounts of a specific antibiotic were placed on agar plates of appropriate media. These plates had previously been streaked with cultures of micro-organisms isolated from the oral cavities of rice rats with extensive periodontal lesions. Since the enterococci seemed to be most closely associated with the periodontal syndrome, influences by the antibiotics upon these micro-organisms were considered most heavily in the selection. The levels of antibiotics chosen were those which caused readily recognizable effects on the growth of the micro-organisms as evidenced in the zone of inhibition.

The experimental subjects were separated at weaning into 5 groups that were as comparable as possible with respect to parentage and sex. The first group of rice rats

---

These studies were supported in part by U.S.P.H.S. research grant D-322 from the National Institute of Dental Research, National Institutes of Health.

Received for publication October 1, 1960.

* Present address: 104 Perkins St., Jamaica Plain 30, Mass.

† Polymyxin B sulfate (Aerosporin^{R}, Chas. Pfizer & Co., Inc.).

‡ Erythromycin glucoheptonate (Ilotycin^{R} glucoheptonate, Eli Lilly and Co.).

§ A benzylpenicillin preparation (Duracillin^{R} fortified, Procaine penicillin G, and buffered crystalline penicillin G, Eli Lilly and Co.).

|| Streptomycin sulfate (Eli Lilly and Co.).

Oxytetracycline hydrochloride (Terramycin^{R} hydrochloride, Chas. Pfizer & Co., Inc.).

was maintained on diet 700 as controls.[2] The second and third groups were fed diet 700 supplemented with 0.033 and 0.198 per cent polymyxin B sulfate, respectively. These levels were selected on the basis of the above in vitro tests, which suggested that amounts of 50–300 units of polymyxin B sulfate needed to be present in the oral cavity at any one time. The polymyxin B sulfate preparation that we used contained 1 unit of activity per 0.16 μg. of weight. Lacking any valid way to determine the size of a food bolus for the rice rat, we assumed a value of 25 mg. From these values, the levels of 0.33 and 1.98 gm/kg of diet (0.033 and 0.198 per cent) were calculated. The fourth and fifth groups were fed diet 700 supplemented with 0.012 and 0.072 per cent erythromycin glucoheptonate. The in vitro tests suggested that 2–12 μg. of erythromycin glucoheptonate would be desirable in the oral cavity at a given time. The commercial preparation used contained 1.0 mg. of active erythromycin glucoheptonate per 1.4 mg. This figure, with the assumption again of a 25-mg. bolus of food, resulted in the levels of 0.12 and 0.72 gm/kg of diet (0.012 and 0.072 per cent). These diets and tap water were provided to the rats ad libitum.

In a second experiment the curative influences of supplements to the diet of 0.05 per cent of a benzylpenicillin preparation, 0.05 per cent oxytetracycline hydrochloride, and a combination of 0.025 per cent of a benzylpenicillin preparation and 0.025 per cent streptomycin sulfate were tested. Rice rats from our stock colony were distributed by litter and sex among 9 groups. Groups 1, 5, and 9 were composed of control subjects that were maintained on ration 700 for 10, 12, and 20 weeks, respectively, before they were sacrificed. The rice rats in groups 2, 3, and 4 were maintained on ration 700 only for the first 10 weeks of the experiment and were then transferred to ration 700 supplemented with one of the antibiotic levels for an additional 10 weeks. The rice rats in groups 6, 7, and 8 were maintained on ration 700 for the first 12 weeks of the experiment and were then transferred to an antibiotic regimen for an additional 8 weeks. The rice rats in groups 2 and 6 received the combination of 0.025 per cent of a benzylpenicillin preparation and 0.025 per cent streptomycin sulfate during the second part of their experimental regimen. The rice rats in groups 3 and 7 received the dietary supplement of 0.05 per cent of a benzylpenicillin preparation, while those animals in groups 4 and 8 received the supplement of 0.05 per cent oxytetracycline hydrochloride.

These experiments lasted for a total of 20 weeks, at which time the rice rats were sacrificed during excessive ether anesthesia, the heads stored in 95 per cent ethanol, skinned, and scored for periodontal lesions by the method of Gupta and Shaw.[3]

In the third experiment, the preventive influences of 0.01 and 0.005 per cent of a benzylpenicillin preparation, 0.01 and 0.005 per cent erythromycin glucoheptonate, and 0.03, 0.01, and 0.005 per cent polymyxin B sulfate were tested. This experiment was conducted in two parts, with the levels of the benzylpenicillin preparation and erythromycin glucoheptonate being evaluated in the 5 groups of the first half and the levels of polymyxin B sulfate in the 4 groups of the second half. In an attempt to improve our assay procedure for the periodontal syndrome in the rice rat, periodic oral examinations were conducted on the subjects in this experiment. The rate of progress of the syndrome among about three-quarters of the subjects was sufficient at 12 weeks on experiment to permit ideal scoring. Therefore, these rice rats were sacrificed after 12 weeks on experiment instead of 20 weeks, as had been used routinely in previous experiments. The remaining quarter of the experimental subjects were sufficiently resistant

even after 20 weeks on experiment that they were discarded as far as this experiment was concerned and continued to determine if and when their periodontal structures would be influenced adversely by the regimen. In this discarding process, if the representative of a litter in the control group was free of grossly detectable evidence of the periodontal syndrome, the entire litter was excluded from the experiment. The same procedures were followed for killing, fixing, and evaluating the periodontal lesions as in the previous experiments.

RESULTS

The results for the influence of the higher amounts of polymyxin B sulfate and erythromycin glucoheptonate used in the first experiment are shown in Table 1. The final body weights and the incidence of lesions in the soft and calcified tissues of the periodontium for the control animals are typical for the general population of rice rats represented by our present colony. As evidenced by the standard errors of the means for the evaluation of the periodontal syndrome in both soft and calcified tissues, this population has a fairly high degree of variability of susceptibility to the periodontal syndrome. This variability is considered by us to be within the range to be expected for the expression of a disease entity of complex etiology in an experimental strain that has been in the laboratory for an insufficient number of generations to permit genetic refinement. On the basis of preliminary tests, the variability within the strain appears to result from constitutional variations from rat to rat and not from a lack of transmission of the necessary microbial flora to the oral cavity of the experimental subjects. Intensive efforts are being made to select and inbreed, in order to develop individual strains that are highly predictable with respect either to proneness to, or resistance to, the periodontal syndrome.

Both levels of polymyxin B sulfate and erythromycin glucoheptonate had no marked influence on the growth and development of the rice rats or on their general appearance, behavior, and rate of survival. There was a suggestion that the higher level of polymyxin B sulfate was approaching a toxic dosage, since both the males and the females grew slightly less rapidly and attained slightly lower final body weights than the controls. These differences, however, were not striking, nor should they be considered to be surprising in view of the very high level fed in the diet.

Both levels of erythromycin glucoheptonate caused highly significant reductions in lesions of the soft tissues and of the hard tissues. In the case of the soft tissues, these reductions constituted practically complete prevention of the periodontal syndrome; only 2 of 19 rice rats in group 4 and 3 of 19 in group 5 had any visually detectable evidence of soft-tissue abnormality. With respect to the calcified tissues of the periodontium, the reductions in the syndrome were not so striking on a percentage basis, although still highly significant from a statistical standpoint. In contrast to the benefit to the soft tissues, no rice rat in groups 4 and 5 was entirely free of bone resorption, even though 33 of the 38 were free of grossly detectable abnormalities of the soft tissues.

Both levels of polymyxin B sulfate caused striking reductions in soft-tissue lesions and lesser reductions in hard-tissue lesions. The reduction for the number of soft-tissue areas was significant at the 1 per cent level, while the reductions for the extent of the soft-tissue lesions and for both the number and extent of hard-tissue lesions were significant at the 5 per cent level. Six of the 17 rice rats in group 2 and 6 of the 19

TABLE 1

PREVENTIVE INFLUENCE OF POLYMYXIN B SULFATE AND ERYTHROMYCIN GLUCOHEPTONATE ON PERIODONTAL SYNDROME IN RICE RAT

GROUP	NO. OF RATS		DIETARY SUPPLEMENT (PER CENT)	AV. FINAL BODY WEIGHT		SOFT-TISSUE LESIONS						CALCIFIED-TISSUE LESIONS					
						Number of Areas			Extent of Areas			Number of Areas			Extent of Areas		
	♂	♀		♂ (Gm.)	♀ (Gm.)	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†
1.....	12	8	None	92.0	61.1	7.8	2.1	.....	10.4+	3.1+	.....	18.1	1.4	.....	19.3+	3.8+	.....
2.....	9	8	0.033 Polymyxin B sulfate	93.9	55.4	1.2	0.5	3.2	0.8+	0.4+	2.5	13.4	0.3	2.2	9.2+	0.7+	2.6
3.....	10	10	0.198 Polymyxin B sulfate	83.0	54.8	1.0	0.3	3.2	0.5+	0.2+	2.8	10.1	0.9	5.0	6.8+	0.5+	3.3
4.....	10	9	0.012 Erythromycin glucoheptonate	87.0	63.0	0.2	0.1	3.6	0.1+	0.1+	2.9	11.2	0.9	4.3	7.2+	0.5+	3.1
5.....	10	9	0.072 Erythromycin glucoheptonate	96.7	60.6	0.4	0.2	3.7	0.3+	0.1+	2.9	10.8	0.9	4.6	7.3+	0.7+	3.0

* SEM = Standard error of the mean.

† CR = Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when from 2.0 to 2.9, moderately significant; when 3.0 or higher, highly significant. The means for experimental groups 2 to 5 are compared with the means for the control rats in group 1.

in group 3 had some evidence of soft-tissue pathology. None of the 36 rice rats in groups 2 and 3 were free of lesions of the mineralized tissues. The incidence of the periodontal syndrome in the rice rats on the lower level of polymyxin B sulfate was not strikingly different from that of the rice rats provided with the high level of polymyxin B sulfate or those provided with the two levels of erythromycin glucoheptonate. However, there were suggestions that the lower level of polymyxin B sulfate was approaching the range of ineffectiveness, while there was no evidence of this for the lower level of erythromycin glucoheptonate.

The results of the second experiment are presented in Table 2. No adverse influence upon general health and body-weight gains was noted in any of the antibiotic groups. The supplementation of diet 700 with 0.05 per cent of a benzylpenicillin preparation or with a combination of 0.025 per cent of a benzylpenicillin preparation and 0.025 per cent streptomycin sulfate for 10 weeks after 10 weeks on diet 700 resulted in these rice rats with an average number of soft-tissue areas and an average extent of soft-tissue areas that were highly significantly less than the comparable averages for their littermates maintained for 20 weeks on diet 700. The supplement of 0.05 per cent oxytetracycline hydrochloride was slightly less effective under the same comparative circumstances. When the groups of rats maintained on the three antibiotic regimens were compared with their controls that were sacrificed at least 10 weeks—the time when the antibiotic administration was begun—the periodontal syndrome in the soft tissues of the experimental rats was noted to be less severe than for the controls but by amounts which were at the borderline of significance. In the comparison of the number of areas of calcified-tissue lesions in the experimental groups and in the 10- and 20-week control groups, no significant differences were noted. This was largely because almost every area of the calcified tissue had been affected to some extent by the tenth week on experiment. However, when the extent of the periodontal syndrome was compared between the 3 antibiotic groups and the 20-week control group, highly significant reductions were noted in all 3 antibiotic groups. However, the average extent in the antibiotic groups was almost identical with that for the 10-week control group. This would indicate that these antibiotic regimens had been able to stop the progress of the lesions of the calcified tissues but had not been adequate to encourage or assist in any repair. In contrast, the soft-tissue lesions during this length of antibiotic therapy had not only been stopped in further progress, but some healing had occurred.

In the remaining groups of the second experiment, where the purified diet had been given for 12 weeks followed by 8 weeks of antibiotic administration, the results were similar. However, the shorter period of antibiotic administration made possible a further separation of the effectiveness of the three antibiotic regimens. The supplement of 0.05 per cent of a benzylpenicillin preparation was the most effective in a curative capacity, while oxytetracycline hydrochloride and the combination of a benzylpenicillin preparation and streptomycin sulfate were definitely inferior.

The results of the third experiment are tabulated in Table 3. On a preventive basis throughout the entire experimental period, levels of a benzylpenicillin preparation and of erythromycin glucoheptonate as low as 0.01 and 0.005 per cent in the diet resulted in highly significant reductions in the development of lesions of both the soft tissues and the calcified tissues of the periodontium. As in earlier studies, the soft tissues

TABLE 2

CURATIVE INFLUENCE OF BENZYLPENICILLIN, STREPTOMYCIN SULFATE, AND OXYTETRACYCLINE HYDROCHLORIDE ON PERIODONTAL SYNDROME IN RICE RAT

Group	No. of Rats		Dietary Supplement (Per Cent)	Av. Final Body Weight		Soft-Tissue Lesions						Calcified-Tissue Lesions					
						Number of Areas			Extent of Areas			Number of Areas			Extent of Areas		
	♂	♀		♂ (Gm.)	♀ (Gm.)	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†
1.....	8	9	None	70.6	47.7	10.9	2.7	1.3	14.2+	3.8+	2.7	24.8	0.7	0.6	32.9+	2.7+	3.0
2.....	6	10	0.025 Benzylpenicillin, 0.025 streptomycin sulfate	86.3	58.3	5.3	1.6	3.7	7.7+	2.4+	3.9	23.8	1.1	1.2	28.7+	2.9+	3.6
3.....	9	9	0.05 Benzylpenicillin	91.6	56.9	4.6	1.5	4.0	8.5+	1.4+	4.0	25.3	0.8	0.2	31.3+	3.7+	3.0
4.....	10	7	0.05 Oxytetracycline hydrochloride	82.1	61.0	7.2	1.9	2.8	8.9+	2.4+	3.7	22.0	2.9	1.1	29.2+	3.8+	3.3
5.....	5	5	None	60.2	49.0	16.1	3.7	0.2	28.3+	7.7+	0.4	26.4	1.1	0.6	49.8+	2.1+	0.3
6.....	4	2	0.025 Benzylpenicillin, 0.025 streptomycin sulfate	77.4	50.0	6.7	4.0	1.9	12.8+	9.2+	1.8	23.7	2.1	0.8	37.3+	9.9+	1.3
7.....	4	4	0.05 Benzylpenicillin	89.0	49.4	4.3	1.1	4.4	5.0+	1.5+	4.6	25.8	0.7	0.3	32.6+	2.9+	3.0
8.....	4	4	0.05 Oxytetracycline hydrochloride	90.0	52.3	10.0	2.7	1.5	15.3+	4.7+	2.3	24.0	1.2	1.1	39.1+	6.6+	1.5
9.....	13	17	None	77.6	54.7	15.3	2.2	.....	32.5+	5.8+	.....	25.5	0.8	.....	51.8+	5.7+	.....

* SEM—Standard error of the mean.

† CR—Critical ratio. The means for groups 1–8 are compared with the means for the control rats in group 9.

TABLE 3

PREVENTIVE INFLUENCE OF LOW LEVELS OF BENZYLPENICILLIN, POLYMYXIN B SULFATE, AND ERYTHROMYCIN GLUCOHEPTONATE ON PERIODONTAL SYNDROME IN RICE RAT

GROUP	NO. OF RATS		DIETARY SUPPLEMENT (PER CENT)	AV. FINAL BODY WEIGHT		SOFT-TISSUE LESIONS						CALCIFIED-TISSUE LESIONS					
						Number of Areas			Extent of Areas			Number of Areas			Extent of Areas		
	♂	♀		♂ (Gm.)	♀ (Gm.)	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†
1.....	9	8	None	60.1	49.4	27.1	0.6	.....	46.6+	3.9+	.....	27.3	0.5	.....	53.4+	4.7+	.....
2.....	8	5	0.01 Benzylpenicillin	66.1	54.8	0.2	0.2	44.8	0.1+	0.1+	11.9	7.3	0.5	28.6	4.5+	0.3+	10.4
3.....	7	3	0.005 Benzylpenicillin	74.5	51.8	0.0	0.0	45.2	0.0	0.0	11.9	8.8	1.1	15.4	5.8+	0.8+	10.0
4.....	6	9	0.01 Erythromycin glucoheptonate	62.5	51.1	0.0	0.0	45.2	0.0	0.0	11.9	8.1	0.6	24.0	5.4+	0.5+	10.2
5.....	8	9	0.005 Erythromycin glucoheptonate	77.1	51.3	0.0	0.0	45.2	0.0	0.0	11.9	7.6	0.6	24.6	4.6+	0.4+	10.4
6.....	10	6	None	65.2	49.0	26.7	1.1	.....	41.3+	4.1+	.....	27.6	0.3	.....	49.9+	5.6+	.....
7.....	10	7	0.03 Polymyxin B sulfate	61.3	39.9	24.4	1.7	1.2	38.4+	4.9+	0.5	25.4	1.4	1.6	48.5+	5.1+	0.2
8.....	10	8	0.01 Polymyxin B sulfate	65.6	41.4	25.7	1.5	0.5	41.3+	4.0+	0.0	26.6	0.6	1.4	49.0+	4.7+	0.1
9.....	8	6	0.005 Polymyxin B sulfate	54.9	48.4	24.1	2.2	1.0	36.5+	4.9+	0.8	25.7	1.3	1.5	42.5+	5.3+	0.7

* SEM—Standard error of the mean.

† CR—Critical ratio. The means for experimental groups 2–5 are compared with those for group 1; the means for experimental groups 7–9 are compared with those for group 6.

were most favorably affected, with almost complete prevention of the periodontal syndrome. While the benefits to the calcified tissues were remarkable, even so, modest scores occurred in practically all experimental subjects. The levels of polymyxin B sulfate used in this experiment (0.03, 0.01, and 0.005 per cent) proved to be incapable of influencing the occurrence of the periodontal syndrome. Although there had been trends in the first experiment that made it seem likely that the lower level of polymyxin B sulfate used was about at the least effective level, it was surprising that the closely similar level used as the highest one in this experiment was entirely ineffective. One possible explanation for this discrepancy may have been the fact that two different batches of polymyxin B sulfate were used for the two experiments that had slightly different activity values.

DISCUSSION

In the two experiments conducted on a preventive basis, a benzylpenicillin preparation, erythromycin glucoheptonate, and polymyxin B sulfate at the highest levels tested were approximately equal to each other in their ability to prevent the development of lesions of the soft tissues of the periodontium and to retard alveolar bone resorption, loosening, and pathologic wandering of the teeth. However, at the lowest levels tested, a benzylpenicillin preparation and erythromycin glucoheptonate continued to be highly effective, while polymyxin B sulfate was ineffective. The lowest levels of a benzylpenicillin preparation and erythromycin gucoheptonate tested were similarly effective and were surprisingly low, indicating that the micro-organisms capable of producing the periodontal syndrome were particularly sensitive to these antibiotics. If the assumption that the food bolus in a rodent as small as a rice rat weighs 25 mg. is approximately correct, the lowest level (0.005 per cent) of these antibiotics in the diet would provide only 1.25 μg. per food bolus.

The levels of a benzylpenicillin preparation and erythromycin glucoheptonate used in the third experiment appear to have been more effective than comparable levels in the first experiment, especially with respect to the lesions of the calcified tissues of the periodontium. This difference is probably explainable on the basis of the shorter assay period of 12 weeks in the third experiment in comparison with 20 weeks in the first experiment. The longer experimental period appears to have provided the opportunity for undetectable incipient lesions to develop sufficiently in the additional 8 weeks to be readily demonstrable in the scoring procedure.

On a curative basis, a benzylpenicillin preparation was found to be more effective than oxytetracycline hydrochloride or a mixture of a benzylpenicillin preparation and streptomycin sulfate. The results in this experiment were much less striking than those in the preventive experiments. While regression of the soft-tissue lesions appeared to have taken place to a definite degree, no evidence of any repair was observed in the alveolar bone, although further progress of the lesions had been greatly retarded.

These studies clearly indicated that there is a strong microbial component in the etiology of the periodontal syndrome in the rice rat. However, it should be emphasized that the principal benefit occasioned by the effective antibiotic was with respect to the soft tissues of the periodontium, where almost complete prevention had been possible, as well as partial repair in curative tests. While the rate of alveolar bone resorption was greatly reduced as a result of effective antibiotic regimens, there was a

strong tendency for the alveolar bone resorption to continue at a reduced but relentless rate, even in the absence of visually detectable lesions of the soft tissues and in the absence of visible oral factors such as calculus formation and food or hair impaction.

SUMMARY

Three hundred and sixty-two rice rats were used in the study of the influence of antibiotics on the prevention and treatment of the periodontal syndrome.

A benzylpenicillin preparation and erythromycin glucoheptonate were highly effective in the prevention of the syndrome, even at levels as low as 0.005 per cent in the diet. Polymyxin B sulfate was effective at high levels but ineffective at comparably low levels.

In curative trials, a benzylpenicillin preparation resulted in a regression of the soft-tissue lesions but was able only to retard the further progress in alveolar bone resorption. Oxytetracycline hydrochloride and a combination of a benzylpenicillin preparation and streptomycin sulfate were less effective than a benzylpenicillin preparation alone in the curative trials.

The B-complex vitamins in the diets were supplied by Dr. Lyon P. Strean, Merck, Sharp & Dohme Research Laboratories, West Point, Pa. The polymyxin B sulfate was supplied by Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

REFERENCES

1. Gupta, O. P., Auskaps, A. M., and Shaw, J. H. Periodontal Disease in the Rice Rat. IV. The Effects of Antibiotics on the Incidence of Periodontal Lesions, *Oral Surg., Oral Med., Oral Path.*, **10**:1169, 1957.
2. Shaw, J. H. Carious Lesions in Cotton Rat Molars. I. The Role of Mechanical Factors Studied by the Extraction of Antagonistic Molars, *J. D. Res.*, **26**:47, 1947.
3. Gupta, O. P., and Shaw, J. H. Periodontal Disease in the Rice Rat. II. Methods for the Evaluation of the Extent of Periodontal Disease. *Oral Surg., Oral Med., Oral Path.*, **9**:727, 1956.

Changes in the Connective Tissue of Rats Fed Toxic Diets Containing Molybdenum Salts

C. A. OSTROM, R. VAN REEN, and C. W. MILLER

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

During the past few years molybdenum has been demonstrated to be an integral part of several enzyme systems.[1] This is presumptive evidence that molybdenum is a required nutrient for animals. More convincing evidence would be the demonstration of a requirement of the mineral for growth or normal development, but this has not been accomplished. Experiments with chicks by Reid, Kurnick, Suacha, and Couch[2] indicated a growth stimulation by molybdenum salts; however, these findings have been questioned because of the sulfate concentration used in the rations. Thus the dietary requirement for molybdenum remains less well established than for some of the other trace elements. Experiments by workers in other areas have provided data which suggest that molybdenum salts may have a cariostatic action. Two of these studies used rats,[3, 4] and two others involved dietary surveys on human populations.[3, 5] In many other studies molybdenum has been shown to exert a toxic effect on animals when fed at elevated levels.[6–8] Some of the toxic effects produced by the administration of molybdenum which have been reported are anemia, diarrhea, fragility of bones, elevated alkaline phosphatase of liver, reduced activity of liver sulfide oxidase, and, more recently, our laboratory has reported the occurrence of mandibular exostoses in rats of the NMRI-D strain.[9] The present paper reports some observations on rats fed toxic diets containing molybdenum and the results of histological examination of dental tissues from these animals.

EXPERIMENTAL METHODS

Albino rats of the NMRI-D strain were used for these experiments. The rats were 21 days of age and between 29 and 51 gm. in weight at the start of the experiment, when they were placed in individual stainless-steel, wire-bottomed cages and maintained in an air-conditioned room. Two groups of animals were employed. The control group was fed a diet containing the following ingredients: "vitamin-test" casein, 20.0 per cent; sucrose, 67.2; U.S.P. XIV salt mixture, 4.0; vitamin mixture, 2.2; and corn oil, 6.6. The test group was fed a toxic diet containing molybdenum, prepared by adding 0.1 gm. of $Na_2MoO_4 \cdot 2H_2O$ to 100 gm. of the basal diet. This amount of sodium molybdate/100 gm of diet provided a level of 400 p.p.m. molybdenum, or 0.4 mMoles of sodium molybdate/100 gm of diet. Distilled water was available ad libitum to both groups of animals.

The opinions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

Received for publication October 6, 1960.

The weight of each rat was recorded weekly. At the termination of the experiment after 5 weeks, the hearts of all animals were punctured and blood samples obtained for the determination of hemoglobin, hematocrit, and white-cell count. At autopsy each animal was examined for mandibular and maxillary exostoses. These tissues were removed from control and experimental animals, stripped of soft tissue down to the periosteum, and fixed in neutral formalin. After examination of the specimens by radiographs and at magnifications of 15 and 60×, the mandibles of two control animals and of four test animals showing exostosis above the masseteric ridge were decalcified, imbedded in paraffin, sectioned in mesiodistal or buccolingual longitudinal plane, and stained with hematoxylin and eosin.

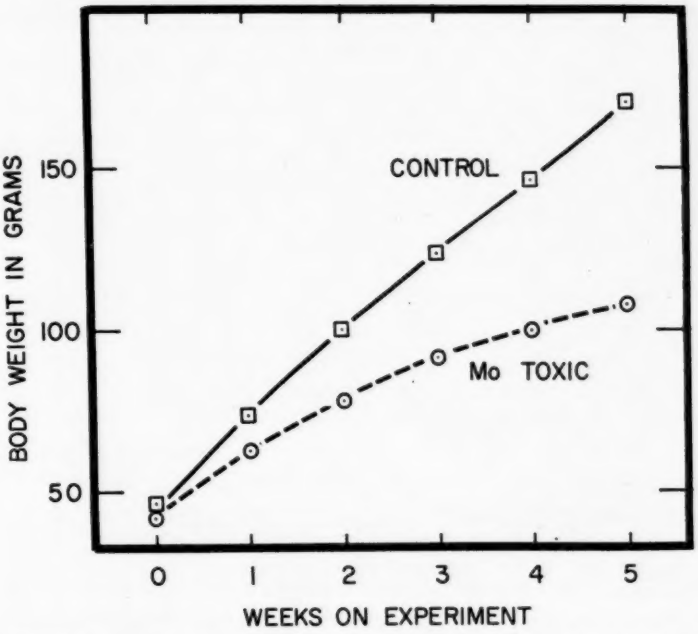


FIG. 1.—Growth response of the NMRI-D rat on a purified diet with and without 400 p.p.m. sodium molybdate supplementation, in terms of body weight.

RESULTS AND DISCUSSION

Growth responses.—The mean weights of the rats fed the diet containing sodium molybdate were markedly less than the control animals (Fig. 1). After 5 weeks on the experimental diets, the control group had a mean weight of 160 gm., in comparison with the molybdenum group, which averaged 109 gm. Ten of the twenty rats on the molybdenum supplementation developed mandibular exostoses. An examination of the weight gains indicated that the rats which formed exostoses were those which also showed the poorest weight gains. The correlation of weight gains with the development of exostoses is shown in Table 1. It should be noted that the rats which did not develop exostoses had a mean body weight of 133.7 gm., which is less than the control group, and thus this subgroup also demonstrated some indications of poisoning. It is

not possible at the present time to determine the reason for the difference in response to molybdenum shown by the two subgroups. Genetic constitution and individual animal differences in the storage of compounds which might mitigate the effects of molybdenum are two possibilities. It is known that molybdenum toxicity can be ameliorated by a number of compounds such as methionine, cystine, thiosulfate, and sodium sulfate. That the first three of the above substances can give rise to inorganic sulfate suggests that inorganic sulfate is the active metabolic antagonist of molybdate.[10] Further studies on the blood or tissue content of these substances might be fruitful.

TABLE 1

CORRELATION OF OCCURRENCE OF MANDIBULAR EXOSTOSES WITH DEVIATION FROM MEAN BODY WEIGHT

MOLYBDENUM TOXIC RATS	NO. OF RATS	MEAN BODY WEIGHT (GM.)	NO. OF RATS WITH DEVIATION FROM MEAN WEIGHT			
			>15 Gm.	−15 to −10 Gm.	−10 to +10 Gm.	>+10 Gm.
With exostoses	10	84.8	7	2	0	1
Without exostoses	10	133.7	1	0	1	8
Total	20	109.3	8	2	1	9

TABLE 2

BLOOD DATA*

DETERMINATION	4 CONTROL RATS	MOLYBDENUM TOXIC RATS		
		7 Rats with Exostoses	8 Rats without Exostoses	All Molybdenum Toxic Rats
Hemoglobin (gm/100 ml)	13.8±0.8	8.4±0.9	13.2±0.4	11.0±0.8
Hematocrit (per cent)	43.7±2.0	29.2±3.4	42.6±1.9	36.4±2.6
MCHC Per cent	31.5±1.5	29.5±2.1	31.2±1.3	30.4±1.2
WBC/mm³	3,350	3,450	3,450	3,450

* Values presented are mean figures ± standard error.

Hematology.—In Table 2 data are presented concerning the values for hemoglobin, hematocrit readings, mean corpuscular hemoglobin concentration, and white-blood-cell count; and, since the weight responses of the rats to molybdenum indicated a distribution which correlated with the occurrence of exostoses, the data on blood also are broken down into subgroups. It was observed that the rats which received the molybdenum supplementation and developed exostoses had hemoglobin concentrations far below that found in the controls. Likewise, the hematocrit values in this subgroup were low and the mean corpuscular hemoglobin concentration a little less than in the controls. The rats receiving molybdenum but which did not form exostoses were not perceptibly different from the control rats. All groups had similar white-blood-cell counts.

Histologic findings.—Radiographic and macroscopic examination showed exostosis on ten of the twenty sets of mandibles; but no cases of gross condylar deformation, molar tipping, or deformation of the incisors, as described in osteolathyrism by Gardner, Dasler, and Weinman,[11] were found.

In microscopic examination, all experimental samples showed large areas of exostosis on the lateral surface of the mandibles, extending to the inferior border and to the angle formed by the ramus, as well as on the coronoid and condyloid processes. The exostoses were superficial to the normal-appearing cortical plate (Fig. 2) and appeared

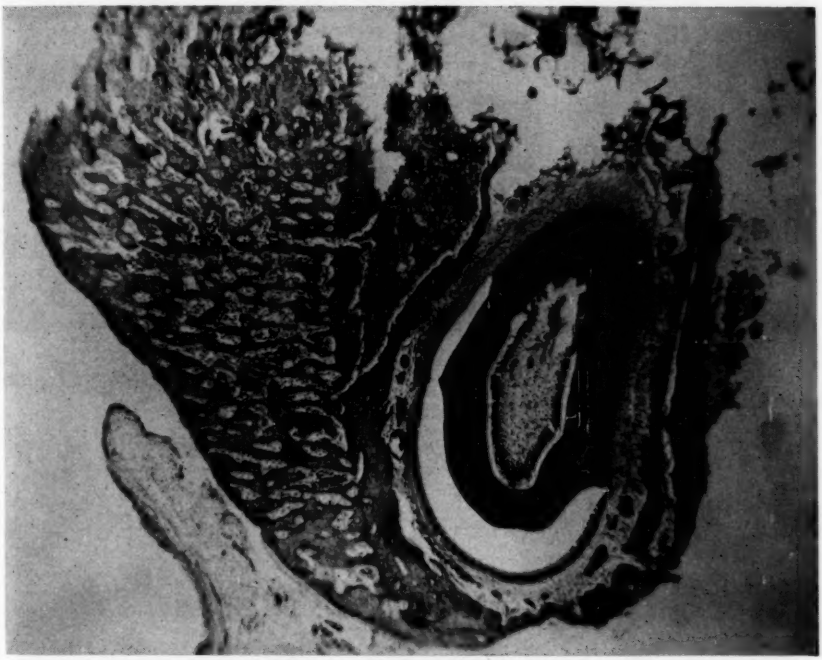


FIG. 2.—Buccolingual longitudinal section of rat mandible below mesial surface of first molar after 35 days on toxic diet containing molybdenum. Exostosis appears superficial to buccal cortical plate. Calciotraumatic line in shown in incisor. (H. and E. stain, mag. ×48.)

trabeculated along the lines of muscle pull. At higher magnification a basophilic area was observed at the surface of the exostoses, and eosinophilic lakes were observed in the insertion site of the masseter muscle (Fig. 3). At 1,900× magnification, the basophilic surface demonstrated basophilic globules (Fig. 4) similar to those reported by Selye[12] and Gardner.[13] Two experimental animals showed moderately small spurlike exostoses extending medially from the inferior aspect of the alveolar crest of the mandibular incisor.

Although no molar malposition was recognized, the supporting tissues showed histologic abnormalities suggestive of mesial rotation of the molars. Spurlike hypercementoses were seen on the molars at the cemento-enamel junction and related to the insertion of the transseptal fibers of the periodontal ligament (Fig. 5). Marked hypercementosis on the mesial surfaces of the apical portion of most molar roots was asso-

FIG. 3.—Insertion site of masseter muscle on rat mandible after 35 days on toxic diet containing molybdenum. Basophilic zone appears at surface of exostosis and eosinophilic lakes appear in fibrous connective tissue. (H. and E. stain, mag. ×90.)

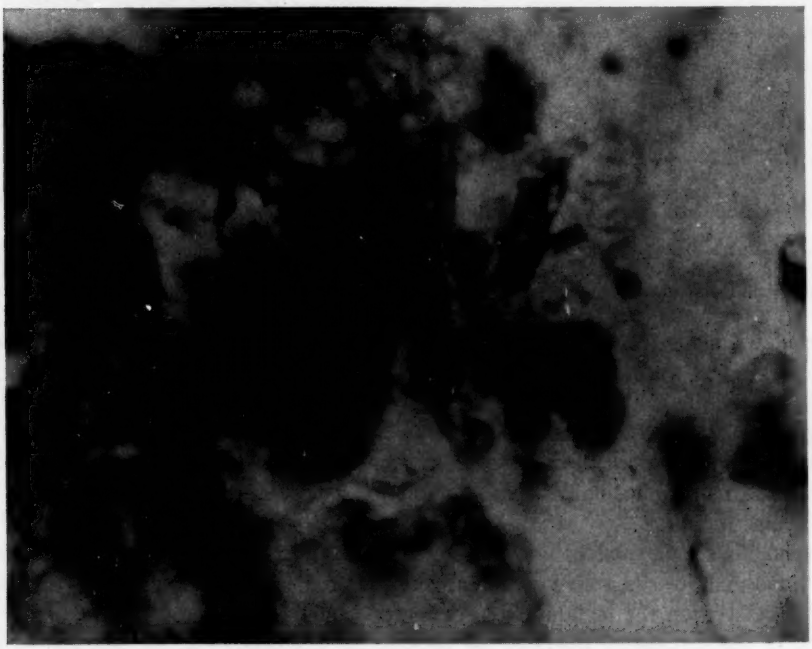


FIG. 4.—Enlarged view of Fig. 2 showing bone granules in basophilic zone on surface of exostosis. (H. and E. stain, mag. ×1,900.)

ciated with tensed- versus relaxed-appearing periodontal fibers and osteogenic activity pathognomonic of tooth movement (Fig. 6). In 56-day-old rats, this degree of hypercementosis would seem significant. At the surface of the lamina dura in areas of tensed periodontal fibers, basophilic bodies similar to Selye's bone globules were seen (Fig. 7). In general, the irregularity and basophilia observed in the incremental lines of the lamina dura and the supporting trabecular bone were suggestive of altered activity. The mandibular marrow appeared normal.

The molar tissues appeared normal. The experimental incisor tissues showed non-

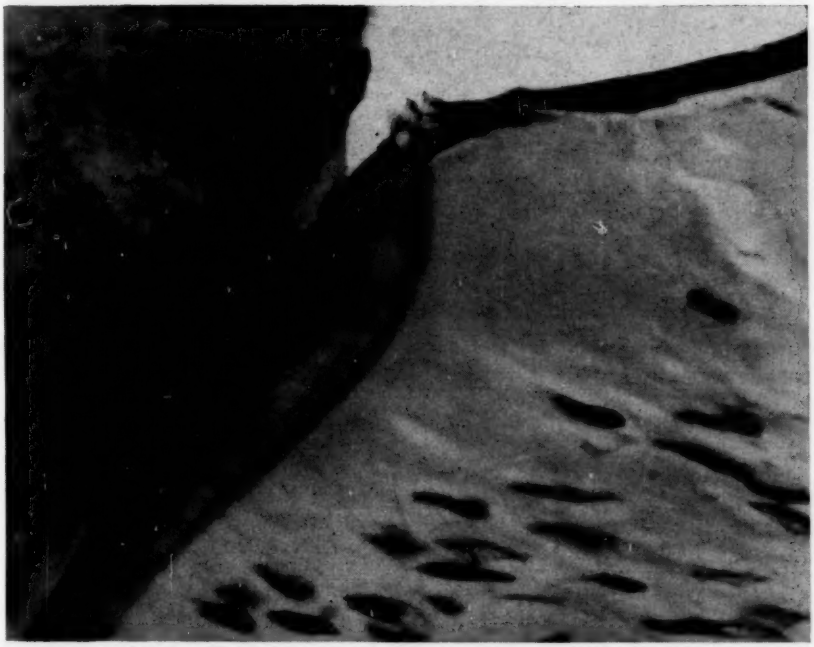


FIG. 5.—Hypercementosis at insertion site of transseptal fibers of periodontal membrane at distal surface of second molar of rat after 35 days on toxic diet containing molybdenum. (H. and E. stain, mag. ×500.)

specific abnormalities. Concentric hypocalcified rings were seen most clearly in cross-sections of the dentin cut near the level of the alveolar crest (Fig. 2). The odontoblastic layer was grossly disarranged in all parts, as seen most clearly in the apical third (Fig. 8), where the granular-appearing odontoblastic cells were interlaced with numerous fibroblasts and lymphocytes, as well as modest numbers of extravascular erthyrocytes and phagocytic cells. Trapped nuclear material was occasionally found in the dentin and predentin of the experimental incisors. No abnormalities were seen in the incisor enamel and ameloblastic tissues.

To a degree, the cementum spurs observed in this study appear similar to Miglani's observation in hypervitaminosis A.[14] The occurrence of Selye's "bone globules" in the lamina dura does not appear to have been reported elsewhere. With these two exceptions, as well as the abnormalities observed in the incisor odontoblastic layer,

FIG. 6.—Hypercementosis on mesial surface of mesial root of mandibular second molar of 56-day-old rat after 35 days on toxic diet containing molybdenum. (H. and E. stain, ×75.)

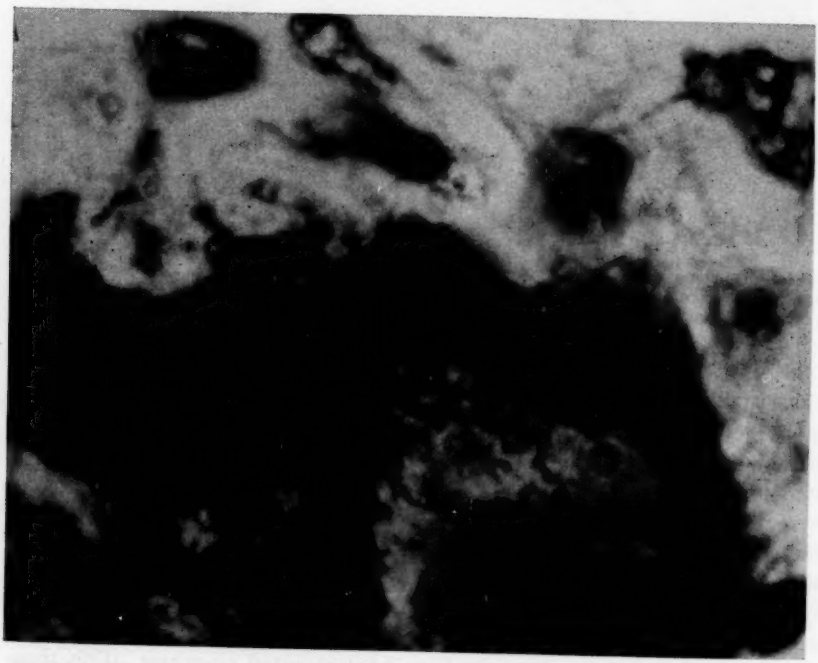


FIG. 7.—Bone granules in basophilic zone in lamina dura at insertion site of tensed fibers of periodontal membrane from Fig. 5. (H. and E. stain, mag. ×1,900.)

the histologic changes observed in this study appear to coincide with the picture of lathyrism described by Gardner.[11, 13, 15–17] The only differences from lathyrism which were observed appeared to be a matter of degree and might be resultant from the strain of rat used or the level of toxicity employed.

While the molybdate intake of these animals was considerably higher than the levels considered relative to caries reduction,[3–5] it would not be illogical to expect that those lower levels might also produce connective tissue abnormalities. As discussed by Selye[12] and Gardner[16] concerning lathyrogenic agents, the stress of caries-inhibiting levels of

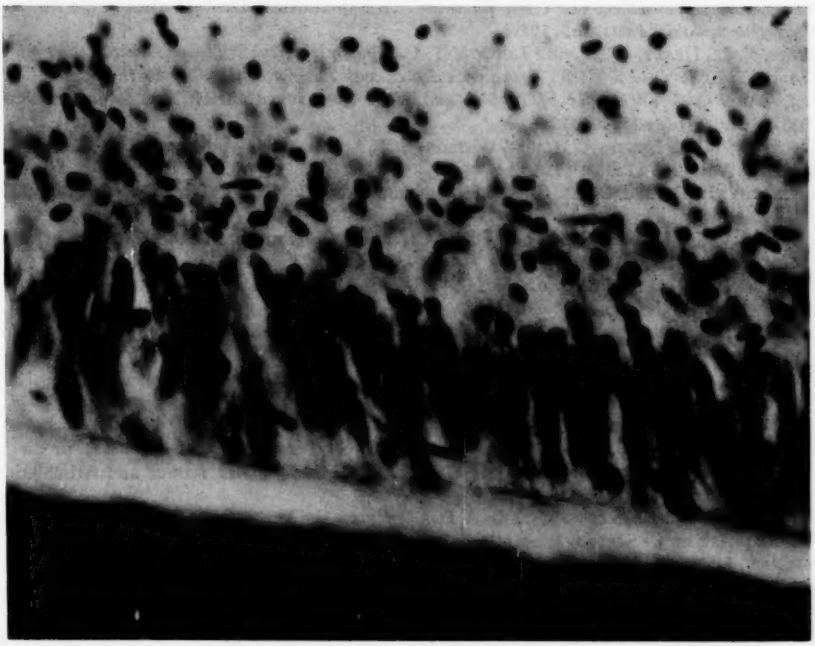


FIG. 8.—Sagittal section in apical third of incisor root. Disorganized odontoblastic layer contains granular-appearing odontoblastic cells interlaced by numerous lymphocytes and fibroblasts, modest numbers of erythrocytes and occasional phagocytic cells. (H. and E. stain, mag. × 250.)

molybdenum might predispose systemic disease and periodontal changes. For these reasons, histologic study of the dental tissues is planned in association with this activity's third study in this field: the cariostatic influence of low-level dietary molybdenum in the NMRI-D rat.

SUMMARY

Molybdenum toxicity was produced in rats of the NMRI-D strain by adding 400 p.p.m. molybdenum as sodium molybdate to a basal purified diet.

Fifty per cent of the animals receiving the molybdenum showed markedly poorer growth responses, low hemoglobin levels, low hematocrit values, and developed mandibular exostoses.

The mandibles and teeth were subjected to histologic examination which indicated that the morphologic changes attendant on molybdenum toxicity resembled lathyrism except for minor details.

REFERENCES

1. Mahler, H. R., and Green, D. E. Metallo-Flavoproteins and Electron Transport, *Science*, **120**:7, 1954.
2. Reid, B. L., Kurnick, A. A., Suacha, R. L., and Couch, J. R. The Effect of Molybdenum on Chick and Poult Growth, *Proc. Soc. Exptl. Biol. Med.*, **93**:245, 1956.
3. Adler, P. Experiments with Albino Rats upon the Caries Protective Effect of Water-borne Molybdenum, *Proc. Orca Cong.*, **14**:48, 1957.
4. Kruger, B. J. The Effect of "Trace Elements" on Experimental Dental Caries in the Albino Rat, *Univ. Queensland Papers*, **1**:3, 1959.
5. Losee, F. L., Healy, W. B., and Ludwig, T. G. *Epidemiological Study of Dental Disease in New Zealand. I. The Dental Caries Prevalence Rates and Their Relationship to the Minerals in the Soil, Water and Vegetables of Napier and Hastings—1959* (NMRI Res. Report MR005.12-5000.01, No. 9, June 24, 1960).
6. Ferguson, W. S., Lewis, A. H., and Watson, S. J. Action of Molybdenum in Nutrition of Milking Cows, *Nature*, **141**:553, 1938.
7. Arrington, L. R., and Davis, G. K. Molybdenum Toxicity in the Rabbit, *J. Nutrition*, **51**:295, 1953.
8. Neilands, J. B., Strong, F. M., and Elvehjim, C. A. Molybdenum in the Nutrition of the Rat, *J. Biol. Chem.*, **172**:431, 1948.
9. Van Reen, R. The Specificity of the Molybdate-Sulfate Interrelationship in Rats, *J. Nutrition*, **68**:243, 1959.
10. Van Reen, R., and Williams, M. A. Studies on the Influence of Sulfur Compounds on Molybdenum Toxicity in Rats, *Arch. Biochem. Biophys.*, **63**: 1, 1956.
11. Gardner, A. F., Dasler, W., and Weinman, J. P. Masticatory Apparatus of Albino Rats in Experimental Lathyrism, *J. D. Res.*, **37**:492, 1958.
12. Selye, H. Recent Progress in Stress Research, *J. D. Med.*, **14**:194, 1959.
13. Gardner, A. F. The Effect of Various Levels of Sweet Peas (*Lathyrus odoratus*) in the Diet, with Five Dietary Supplements, on the Growth of the Mandibular Ramus and Articulation in Young Rats, *J. D. Res.*, **38**:805, 1959.
14. Miglani, O. C. The Effect of Vitamin A Deficiency on the Periodontal Structure of Rat Molars with Emphasis on Cementum Resorption, *Oral Surg., Oral Med. & Oral Path.*, **12**:1372, 1959.
15. Gardner, A. F. The Effects of Various Levels of Sweet Peas (*Lathyrus odoratus*) in the Diet with Protein and Aminoacid Supplementation, on the Alveolar Bone of Young Rats, *Oral Surg., Oral Med. & Oral Path.*, **12**:876, 1959.
16. ———. Influence of Injury and Adaptation of the Periodontal Ligament to Pathologic Changes during Experimental Lathyrism, *J. Periodont.*, **30**:253, 1959.
17. ———. Morphologic Study of Oral Connective Tissue in Lathyrism, *J. D. Res.*, **39**:24, 1960.

Additional Studies on Fluoride Uptake by Enamel from Certain Dental Materials

RICHARD D. NORMAN, JAMES R. PLATT, RALPH W. PHILLIPS, and MARJORIE L. SWARTZ

Department of Dental Materials, Indiana University School of Dentistry, Indianapolis, Indiana

It has been shown that acid solubility of both powdered and intact enamel is altered by contact with certain dental materials.[1] A marked reduction in the solubility was induced by some of these materials, and the phenomenon appeared to be related to the presence of fluorine in these materials. Further investigations revealed that fluorine was released by the materials and that the fluorine content of powderd enamel subsequently increased.[2] There appeared to be definite relationships between the quantity of fluorine contained in the material, the amount of fluorine taken up by powdered enamel, and the decrease in solubility.

Although powdered enamel provides a satisfactory screening method, the changes induced are generally magnified because of the greater surface area exposed and the lack of an organic matrix. Thus it was felt that the use of intact enamel would make possible a more practical and realistic means of assessing the influence of the materials and predicting their possible clinical behavior. Hence this present study was conducted to investigate the effects of various materials on the fluorine content of intact enamel surfaces.

MATERIALS AND METHODS

In general, the procedure consisted of utilizing one half of a tooth as the control, while the restorative material was applied to the other half. Through chemical analysis of enamel taken from the two areas, it was possible to determine whether contact with the material had produced a change in the fluorine content.

Freshly extracted caries-free cuspid teeth were employed. Prior to use, they were carefully cleaned and buffed with levigated alumina to remove any debris. They were immediately placed in water and never permitted to dehydrate.

Since Brudevold, Gardner, and Smith[3] established that the fluoride content of teeth is greater at the outer surface and decreases as the dentino-enamel junction is approached, it was mandatory that the thickness of enamel taken for analysis be controlled. Thus a guard was designed which, when fitted to a straight dental handpiece and used with a specific diamond cutting instrument, permitted a uniform depth of

This research was supported in part by a grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-391.

Paper read at the thirty-eighth annual meeting of the International Association for Dental Research, Chicago, Illinois, March, 1960. Portions of the paper taken from a thesis by the senior author in partial fulfilment of the requirements for the M.S.D. degree, Indiana University, 1961.

Received for publication October 10, 1960.

enamel (0.4 mm.) to be removed. Repeated cuts with the instrument proved the operator's ability to control this factor. The grindings were collected by means of a stream of glass-distilled water which was directed onto the wheel and tooth during the cutting operation.

Initially, a layer of enamel from one half of the labial and the adjacent proximal surface was removed and collected. After drying and weighing, the fluorine content was determined, and those data served as the control for the particular tooth. The test material was then applied to the remaining labial and proximal portions of the tooth. The tooth, with the restorative material in place, was stored in distilled water for 2 weeks. The storage temperature was 37° C., and the water was changed daily. At the end of this time the dental material was removed and the underlying layer of enamel separated for analysis.

TABLE 1

Material	Company	Material	Company
S. S. White filling porcelain	S. S. White Dental Manufacturing Co.	Silico-phosphate cement (Kryptex)	S. S. White Dental Manufacturing Co.
Berylite	W. V. B. Ames Co.	Zinc oxide	Merck & Co., Inc.
FluorOn	American Consolidated Dental Manufacturing Co., Inc.	Eugenol	Kerr Manufacturing Co.
Experimental resin+5% NaF*	L. D. Caulk Co.	Experimental zinc oxide and eugenol+2% NaF*	L. D. Caulk Co.
Experimental resin+2% NaF*	L. D. Caulk Co.	Kadon	L. D. Caulk Co.
Zinc phosphate cement	S. S. White Dental Manufacturing Co.	Calcium hydroxide paste	L. D. Caulk Co.
		Copalite	Harry J. Bosworth Co.
Experimental zinc phosphate cement+10% CaF_2*	L. D. Caulk Co.	Sodium fluoride	J. T. Baker Chemical Co.

* Per cent fluoride added to powder.

The analytical procedure was one involving the microdistillation of the samples, as described by Singer, Armstrong, Venkateswarlu, Sheck, and Cummins.[4] Fluorine was measured by a modification of Megregian's spectrophotometric procedure.[5]

Control tests were first run to ascertain the normal variation in the fluorine content between the two halves of the tooth. The enamel from the right and left halves of 25 untreated teeth was compared. The difference was determined for each tooth, and the average per cent difference was recorded. It was found that, although there was great variation from one tooth to another, the average difference in the fluorine content of enamel taken from the same tooth was less than 9 per cent.

The materials used in the investigation are shown in Table 1. They were manipulated in accordance with accepted procedures. Before application of the material the tooth surface was always dried by means of compressed air.

A variable included was the effect of a cavity varnish placed on the tooth surface prior to application of the test material. Certain representative materials and a 24-hour treatment of a 2 per cent sodium fluoride solution were used in conjunction with the cavity varnish.

The inherent variation encountered in individual teeth, as revealed by the data se-

cured on the controls (Table 2), emphasized that a large number of specimens should be employed. Therefore, a minimum of 10 teeth, and in most instances 20 or more, were used with each material. The data thus represent results obtained from over 550 teeth and 2,800 spectrophotometric determinations.

RESULTS

The mean results are seen in Table 3, and the individual data of representative materials are shown in Tables 4 and 5. Ames's Berylite cement was the only material that produced a definite reduction in the fluorine content of enamel. The results of the effects of Copalite (the cavity varnish), Kadon, zinc phosphate cement, calcium hydroxide, and the non-fluoride zinc oxide and eugenol mixtures were within the range of individual tooth variation as determined by the controls. The experimental zinc phosphate cement containing 10 per cent calcium fluoride induced a 33 per cent fluorine increase. Likewise, when sodium fluoride was added to zinc oxide and eugenol, there was a 46 per cent increase in the fluorine content of the enamel. Tooth surfaces in contact with FluorOn (a resin containing sodium and stannous fluoride) and the teeth immersed in 2 per cent sodium fluoride for 24 hours exhibited the largest increases in

TABLE 2

VARIATION OF FLUORIDE WITHIN INDIVIDUAL TEETH (CONTROLS)

Tooth No.	Side 1 (p.p.m.)	Side 2 (p.p.m.)	Difference (p.p.m.)	Per Cent Difference
1	92	107	+ 15	+16
2	94	63	− 31	−33
3	150	104	− 46	−31
4	73	82	+ 9	+12
5	628	629	+ 1	0
6	72	99	+ 27	+38
7	594	634	+ 40	+ 7
7	78	70	− 8	−10
9	92	122	+ 30	+33
10	431	317	−114	−26
11	68	104	+ 36	+53
12	84	116	+ 32	+38
13	35	30	− 5	−14
14	132	87	− 45	−34
15	116	132	+ 16	+14
16	56	83	+ 27	+48
17	65	68	+ 3	+ 5
18	119	150	+ 31	+26
19	41	44	+ 3	+ 7
20	85	104	+ 19	+22
21	127	121	− 6	− 5
22	61	74	+ 13	+21
23	196	188	− 8	− 4
24	138	165	+ 27	+20
25	71	85	+ 14	+20
Av.	148	151	+ 3	+ 9
St. Dev.	152	152	.....	24
t	0.052			

fluoride uptake. A 239 per cent increase in fluorine content occurred in enamel which had been exposed to silicate cement, while use of the sodium fluoride resins and Kryptex resulted in increases of approximately 100 per cent. (Fluoride fluxes are used in the manufacturing of silicate and silico-phosphate cements.)[6]

The effect of these materials on enamel which had first been coated with the cavity varnish can be seen in Table 6. The individual results of a representative material are shown in Table 7. Its use did not markedly affect the results with the following materials: zinc phosphate cement, calcium hydroxide paste, zinc oxide and eugenol + 2 per cent NaF, and the fluoride-containing resins. However, the uptake of fluorine from the remaining materials was greatly reduced when Copalite was used. The per cent fluoride uptake by enamel from the silicate cement and 2 per cent sodium fluoride solution was

TABLE 3

Material	Per Cent Change in Fluoride	Material	Per Cent Change in Fluoride
Berylite	−22	Zinc oxide and eugenol+2% NaF	+ 46
Copalite	− 7	Resin+5% NaF	+ 93
Kadon	− 6	Kryptex	+ 99
Zinc phosphate cement	+ 2	Resin+2% NaF	+103
Zinc oxide and eugenol	+10	Silicate	+239
Calcium hydroxide paste	+11	FluorOn	+304
Zinc phosphate+10% CaF_2	+33	2% NaF solution—24 hours	+365

TABLE 4

VARIATION OF FLUORIDE WITHIN INDIVIDUAL TEETH

(Zinc Oxide+2% NaF and Eugenol)

Tooth No.	Control Side (p.p.m.)	Experimental Side (p.p.m.)	Difference (p.p.m.)	Per Cent Difference
1	131	174	43	33
2	121	328	207	171
3	180	83	− 97	− 54
4	205	199	− 6	− 3
5	195	274	79	41
6	215	397	182	85
7	240	243	3	1
8	511	457	− 54	− 11
9	180	280	100	56
10	131	312	181	138
11	257	268	11	4
12	90	196	106	118
13	200	356	156	78
14	260	385	125	48
15	357	403	46	13
Av.	218	290	67	48
St. Dev.	101	98	.....	58
t	7.3			

TABLE 5

VARIATION OF FLUORIDE WITHIN INDIVIDUAL TEETH

(Silicate)

Tooth No.	Control Side (p.p.m.)	Experimental Side (p.p.m.)	Difference (p.p.m.)	Per Cent Difference
1	215	314	99	46
2	99	382	283	286
3	150	264	114	76
4	221	653	432	195
5	112	513	401	358
6	98	207	109	111
7	92	299	207	225
8	183	547	364	199
9	131	350	219	167
10	57	282	225	395
11	61	345	284	466
12	52	254	202	388
13	113	259	146	129
14	85	242	157	185
15	180	453	273	152
16	86	346	260	302
17	150	223	73	49
18	179	660	481	269
19	113	879	766	678
20	91	294	203	223
21	70	281	211	301
22	93	474	281	302
23	89	496	407	457
24	128	275	147	115
25	162	356	194	120
26	158	287	129	82
27	81	228	147	181
Av.	120	384	252	239
St. Dev.	46	158	.....	149
t	8.4			

TABLE 6

Material	Per Cent Change in Fluoride		Material	Per Cent Change in Fluoride	
	Without Copalite	With Copalite		Without Copalite	With Copalite
Berylite	−22	+ 5	Kryptex	+ 99	+ 13
Zinc phosphate cement	+ 2	− 6	Resin+2% NaF	+103	+101
Calcium hydroxide paste	+11	+ 4	Silicate	+239	+104
Zinc phosphate+10% CaF_2	+33	− 4	FluorOn	+304	+382
Zinc oxide and eugenol+2% NaF	+46	+45	2% NaF solution—24 hours	+365	+141

reduced approximately 60 per cent when the cavity varnish was employed. On the other hand, with Ames's Berylite the loss of fluoride from enamel was prevented by the use of the cavity liner.

DISCUSSION

With few exceptions, the data obtained with intact enamel surfaces compare with those found with powdered enamel.[2] However, as might be expected, the magnitude of fluoride change was not so great with the intact surface. The comparative data are summarized in Table 8. FluorOn and silicate cement produced the greatest change in the

TABLE 7

VARIATION OF FLUORIDE WITHIN INDIVIDUAL TEETH

(Resin+2% NaF+Copalite)

Tooth No.	Control Side (p.p.m.)	Experimental Side (p.p.m.)	Difference (p.p.m.)	Per Cent Difference
1	196	615	419	214
2	135	280	145	107
3	187	257	70	37
4	127	312	185	146
5	139	383	244	175
6	140	186	46	33
7	63	120	57	90
8	63	135	72	114
9	79	179	100	127
10	155	230	75	48
11	51	105	54	108
12	52	84	32	62
13	175	451	276	158
14	169	234	65	38
15	112	158	46	41
16	72	157	85	118
Av.	121	242	123	101
St. Dev.	49	126	.....	53
t	13.0			

TABLE 8

Material	Per Cent Change in Fluoride		Material	Per Cent Change in Fluoride	
	Intact Enamel	Powdered Enamel		Intact Enamel	Powdered Enamel
Berylite	−22	− 26	Resin+2% NaF	+103	+ 51
Zinc phosphate cement	+ 2	− 35	Silicate	+239	+593
Zinc phosphate+10% CaF_2	+33	− 19	FluorOn	+304	+506
Resin+5% NaF	+93	+ 66	2% NaF solution	+365*	+128†
Kryptex	+99	+128			

* 24 hours.

† 16 minutes.

fluorine content of both powdered and intact enamel, and generally a comparable relationship was noted with the other materials. These changes in the fluorine content of intact enamel correlate well with data previously obtained on calcium solubility[1, 7] (Table 9). It is interesting to note that Berylite—a material which habitually increased enamel solubility—reduced the fluorine content of the tooth. Neither the fluoride change nor the solubility of enamel was greatly influenced by the zinc phosphate cement. Although there was an increase in the fluorine content of the teeth treated with the zinc phosphate cement containing 10 per cent calcium fluoride, there was no effect

TABLE 9

Material	Per Cent Change in Fluoride	Per Cent Change in Calcium Solubility	Material	Per Cent Change in Fluoride	Per Cent Change in Calcium Solubility
Berylite	−22	+20	Resin+5% NaF	+ 93	−17
Copalite	− 7	+ 5	Kryptex	+ 99	−14
Kadon	− 6	+19	Resin+2% NaF	+103	−29
Zinc phosphate cement	+ 2	+ 7	Silicate	+239	−28
Zinc oxide and eugenol	+10	+13	FluorOn	+304	−53
Zinc phosphate+10% CaF_2	+33	+ 2	2% NaF solution—24 hours.	+365	−60
Zinc oxide and eugenol+2% NaF	+46	−16			

TABLE 10

Material	Per Cent Change in Fluoride		Per Cent Change in Calcium Solubility	
	Without Copalite	With Copalite	Without Copalite	With Copalite
Berylite	− 22	+ 5	+20	+ 6
Resin+2% NaF	+103	+101	−29	−39
Silicate	+239	+104	−28	−19
FluorOn	+304	+382	−53	−45
2% NaF solution—24 hours.	+365	+141	−60	− 7

on solubility. Likewise, Kadon and zinc oxide and eugenol mixtures produced some increase in the calcium solubility of enamel but no appreciable fluoride change. All the other materials resulted in both an uptake of fluorine and a reduction in acid solubility. Furthermore, it should be noted that those materials which produced the greatest increase in fluorine also induced the greatest reduction in enamel solubility.

Previously it has been shown that the effect of the cavity varnish varied with the particular restorative material employed.[8] Copalite did not alter the influence of the resins, whereas the other types of materials were generally less effective when the liner was employed. This may have been due to the fact that the liner was soluble in the resin monomer. A comparison of these data with the solubility data (Table 10) again points out the close relationship between fluorine uptake and the reduction in solubility. This relationship is better illustrated in Figure 1. The increase in fluoride content of the

tooth has been plotted against the change in solubility as induced by each material. When the fluorine content was increased, there was a reduction in the acid solubility of the intact enamel surfaces.

SUMMARY

The fluorine uptake by intact enamel surfaces from certain dental materials has been studied. These data were correlated with those found in a previous investigation using powdered enamel, as well as with studies on enamel solubility as it is affected by various materials. The results were as follows: Most of the materials tested produced a measurable change in the fluorine content of intact enamel. Generally, the data from powdered enamel and intact enamel were in agreement. Copalite had no effect on the fluorine uptake from resins or from a zinc oxide containing 2 per cent sodium fluoride, but the

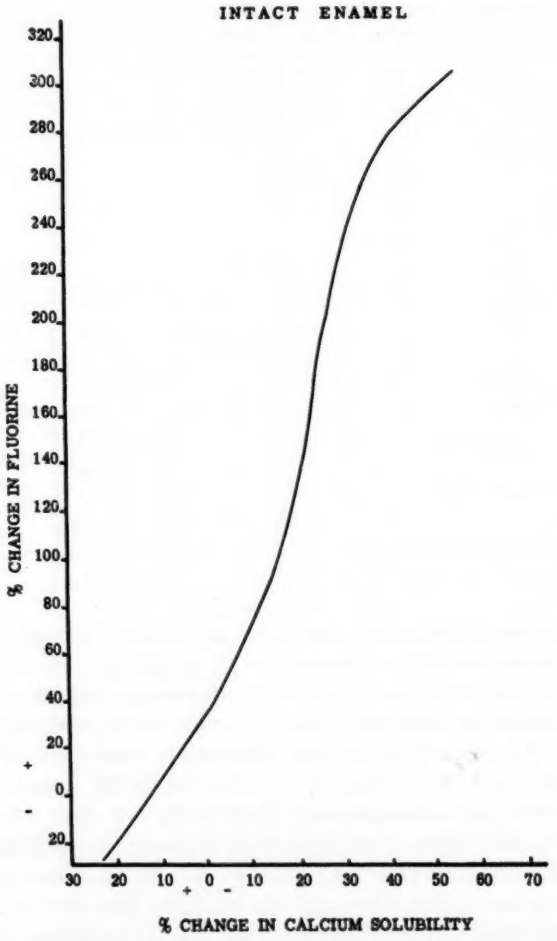


FIG. 1.—Change in calcium solubility plotted against change in fluoride content of intact enamel

varnish did reduce the effectiveness of the other fluoride-containing materials. A definite relationship was found between fluoride change and acid solubility of intact enamel.

REFERENCES

1. PHILLIPS, R. W., and SWARTZ, M. L. Effect of Certain Restorative Materials on the Solubility of Enamel, *J.A.D.A.*, **54**:623, 1957.
2. NORMAN, R. D., PHILLIPS, R. W., and SWARTZ, M. L. Fluoride Uptake by Enamel from Certain Dental Materials, *J. D. Res.*, **39**:11, 1960.
3. BRUDEVOLD, F., GARDNER, D. E., and SMITH, F. A. The Distribution of Fluoride in Human Enamel, *J. D. Res.*, **35**:420, 1956.
4. SINGER, L., ARMSTRONG, W. D., VENKATESWARLU, P., SHECK, J., and CUMMINS, N. Analysis of Fluoride in Plasma and Serum, *J. D. Res.*, **37**:69, 1958.
5. MEGREGIAN, S. Rapid Spectrophotometric Determination of Fluoride with Zirconium-Eriochrome R Lake, *Anal. Chem.*, **26**:116, 1954.
6. SKINNER, E. W., and PHILLIPS, R. W. *The Science of Dental Materials*, p. 244. 5th ed. Philadelphia: W. B. Saunders Co., 1960.
7. PHILLIPS, R. W., and SWARTZ, M. L. Unpublished data.
8. SWARTZ, M. L., and PHILLIPS, R. W. Permeability of Cavity Liners to Certain Agents. *J. D. Res.*, **39**:1232, 1960.

Eruption of Irradiation-produced Rootless Teeth in Monkeys

JOSEPH M. GOWGIEL

Department of Anatomy and Walter G. Zoller Memorial Dental Clinic, University of Chicago

The mechanism of tooth eruption has been the subject of several investigations, and a number of theories attempting to explain this phenomenon have been proposed. Massler and Schour[1] summarized the theories of tooth eruption up to the time of their report and, on the basis of their review and their own investigations, concluded that the eruption of teeth is apparently related to the degree of vascularity of the periapical tissues. In a recent publication, Bryer[2] supported this view. However, Miller[3] and Taylor and Butcher[4] did not find the eruption rate to be altered by fairly considerable alteration of blood flow to the periapical tissues. O'Brien, Bhasker, and Brodie[5] reported on serial histologic sections through the first molar region of *ia* and heterozygous rats and were of the opinion that eruption was probably the result of growth of the tooth follicle. Engle[6] has shown that the connective tissue ground substance overlying erupting teeth is altered in its histochemical staining reaction and thought to be depolymerized. Baume, Becks, and Evans[7, 8, 9] in a series of experiments demonstrated the selective action of growth hormone and thyroxin on tooth eruption and were of the opinion that it was a process of growth and differentiation of maturation. Notwithstanding these investigations, the phenomenon of tooth eruption is still incompletely understood.

This report is based upon a serendipitous finding in an experiment[10] designed to study the pathogenesis of radio-osteonecrosis. In that experiment it was observed that crowns without any root formation erupted into the oral cavity.

MATERIALS AND METHODS

Eighteen *Macacus rhesus* monkeys were subjected to fractionated external irradiation to the mandible and maxilla. The animals reported in this paper were irradiated with dose levels of 4,500, 5,500, and 7,500 r and lived up to 3¼ years post-irradiation. They were irradiated 5 days a week to alternating sides of the face. The length of irradiation treatment varied from 5½ to 12½ weeks, depending on the dose administered. The physical factors of irradiation were: 250 KVP, 15 ma, target-skin-distance, 50 cm., half-value layer, 1.5 mm. Cu, dose rate of 44r/min. The animals were killed with an overdose of nembutal. Tissues were fixed in 10 per cent neutral formalin and decalcified in formic acid–sodium formate solution. Tissues were imbedded in celloidin, cut at 8–10 μ, and stained with hematoxylin-eosin-azure II.

This investigation was supported in part by U.S.P.H.S. research grant C-1442 from the National Cancer Institute, National Institutes of Health.

Received for publication October 11, 1960.

The animals varied in age from 14 to 28 months and therefore had a complete deciduous dentition. Pre-irradiation roentgenographs revealed the permanent teeth to be in various stages of development, with the third molar tooth not yet evident (Fig. 1). Eruption of the teeth was observed by means of lateral jaw roentgenographs taken at regular intervals.

RESULTS

At all dose levels, the irradiation completely inhibited further crown and root development and also destroyed the anlage of the third molar tooth (Fig. 2); therefore, the size and shape of the permanent teeth were dependent on their state of development at the time of irradiation. Calcification of the crown matrix was not inhibited, as can be seen in Figures 1 and 2, in which only cusp tips were calcified at the beginning of irradiation, and they continued to calcify during and after irradiation. Some crowns were only partially formed. The maxillary permanent teeth always had some root formation, usually short and thin (Fig. 3), while the mandibuar teeth were completely rootless. This is in striking contrast to the dentition of a normal, non-irradiated monkey (Fig. 4).

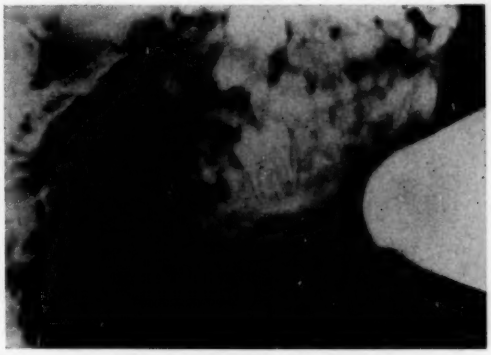


FIG. 1.—Pre-irradiation roentgenograph. Note forming molars and bicuspids

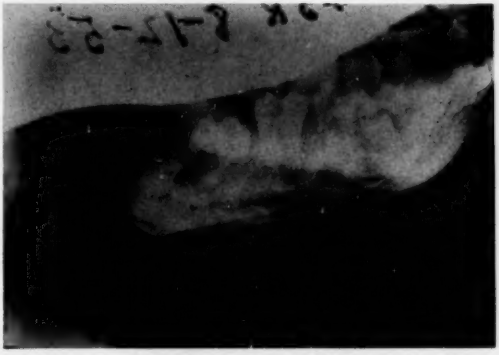


FIG. 2.—Same animal as in Fig. 1. One year post-irradiation, 5500r. Note complete cessation of tooth development.

The pulp chamber of all teeth was diminished in size, and the apex closed in an almost linear manner at the level of maximal tooth development at the time of irradiation. The pulp chamber of the irradiated teeth appeared to close at a more rapid rate than in the normal teeth and was completely closed before they erupted a short distance. Layers of dense bone were usually formed under erupting crowns (Fig. 5). Most of the crowns erupted, but a few of them did not (Fig. 6). In these cases, the crown matrix calcified, and the pulp chamber slowly filled in, as in the crowns that erupted. The few crowns that did not erupt occurred in two of the animals that received 7,500 and 8,500 r and lived $3\frac{1}{4}$ and $1\frac{3}{4}$ years post-irradiation. This ankylosis was not observed in lower irradiation dose levels, even though the animals lived for the same lengths of time.

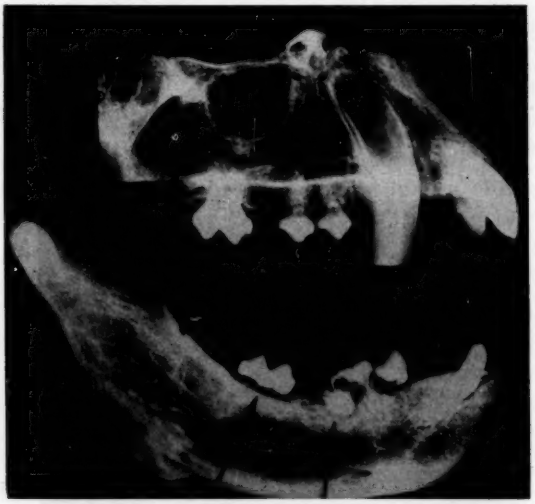


FIG. 3.—Same animal as in Figs. 1 and 2, $3\frac{1}{4}$ years post-irradiation, 5500r. Teeth have been in occlusion for 18 months. Note rootless molar and bicuspids (*arrow*).

FIG. 4.—Normal, non-irradiated monkey. Compare with Fig. 3

The roots of the deciduous molars resorbed in the normal manner. The aberrant size of the permanent bicuspids did not alter this process. The eruption rate of the partially formed permanent teeth appeared to be somewhat retarded, but the degree of retardation was not precisely determined. The partially formed teeth eventually erupted into full occlusion (Fig. 7), and a few partially formed crowns were attached only to the gingiva and were in functional occlusion for 18 months without any evidence of looseness (Fig. 3).

The irradiation produced profound histologic changes in the dental structures. The enamel was removed by the decalcification procedure and therefore could not be examined. There was an abrupt transformation of odontoblasts and dentin from the normal pre-irradiation appearance to the abnormal, irradiation-produced appearance (Fig. 8). The normal tall, columnar odontoblasts and closely packed dentinal tubules changed rather abruptly to low cuboidal odontoblasts and widely spaced dentinal tubules. This appearance was maintained for about 30–50 cells. It then changed abruptly to polygonal-shaped cells and osteodentin. Enamel formation always ended at the junction of the normal dentin and osteodentin, regardless of the crown size

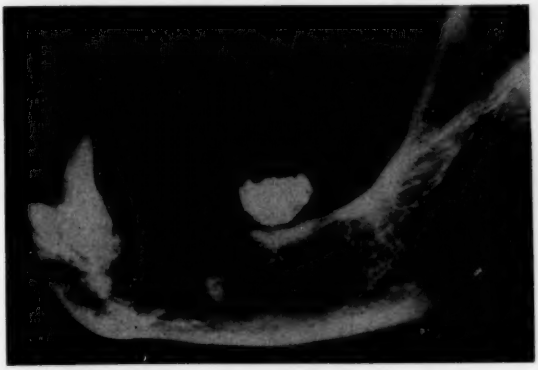


FIG. 5.—Two and three-quarter years post-irradiation. 6500r. Rootless molar erupted and in occlusion. Note dense bone formation under the crown.

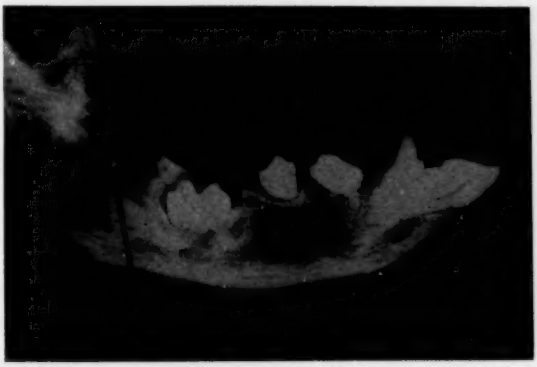


FIG. 6.—Three and one-quarter years post-irradiation, 7500r. Unerupted rootless molar. The distal root of the first bicuspid did not form, while the mesial root was out of the field of irradiation and did form. Note radio-osteonecrotic lesion in ascending ramus (*arrow*).

attained at that time. The epithelial root sheath was not found in all sections, but, when present, it terminated on the osteodentin near its junction with the normal dentin. The outer surface of the osteodentin is covered by cementum which is often very cellular. The follicular sac is attached to the cementum.

As osteodentin formation continues, the trabecular character of its early stages changes to that of a solid mass of calcified tissue with only a few interspersed cellular areas. Concomitantly, the soft tissue undergoes a change from that of pulp tissue to that resembling fibrotic marrow tissue (Fig. 9). In a few molar teeth that did not

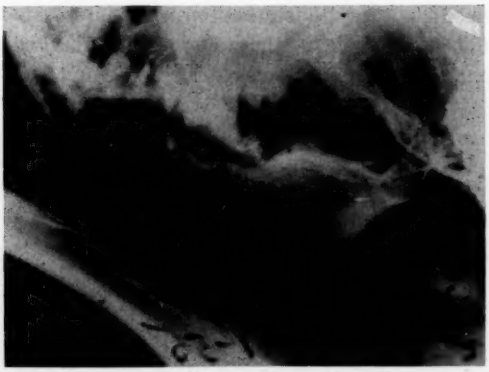


FIG. 7.—Two and three-quarter years post-irradiation, 4500r. Molar teeth in occlusion, no root formation on second molar (*arrow*).

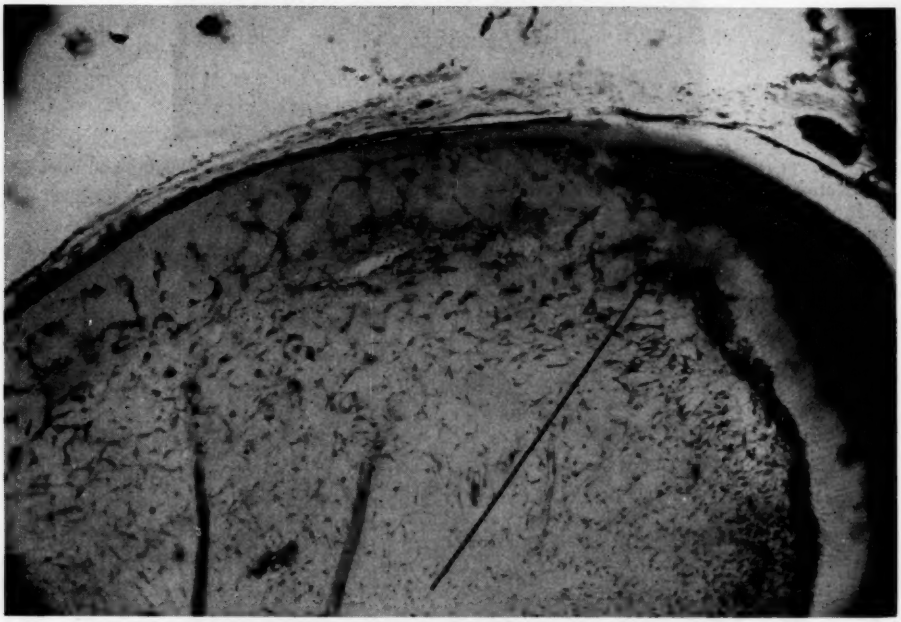


FIG. 8.—Five and one-half weeks post-irradiation, 9500r. Magnification ×110. Irradiation-produced osteodentin. Odontoblasts end abruptly at the junction of normal and abnormal dentin (*arrow*).

erupt, this metaplasia progressed to the point where bone trabeculae were in continuity with the osteodentin and alveolar bone via the pulp chamber (Fig. 10). In these teeth the soft tissue of the pulp chamber had the character of fibrous bone marrow tissue, and the transformed odontoblasts resembled osteoblasts.

Without root formation, the follicular sac did not differentiate into the periodontal ligament but, instead, maintained its attachment to the cemental surface of the crown regardless of the shape or size of the crown. The connective tissue fibers of the sac were relatively loose, cellular, and arranged parallel with the crown. The more lateral fibers of the sac were attached to the surrounding alveolar bone. No changes were

FIG. 9.—Two years post-irradiation, 8500r. Magnification ×255. Pulp tissue is fibrotic; odontoblasts are absent. Osteodentin in continuation with normal dentin is evident (*arrow*).

noted in this arrangement as the tooth continued to erupt until the gingival tissue was reached. At this stage of eruption the more superior fibers of the follicular sac appeared to be continuous with the gingival fibers and were arranged more perpendicular to the crown. Figure 11, which is the second bicuspid tooth of roentgenograph Figure 6, shows that the fibers of the follicular sac are attached to the alveolar crest and gingiva. There is one area of an aborted root formed by osteodentin, and attached to it is a differentiated periodontal ligament. This bicuspid has been in functional occlusion for about 14 months without any evidence of looseness. Figure 12, which is the mandibular molar tooth shown in roentgenograph Figure 3, is a tooth that was in functional occlusion for 18 months without any evidence of looseness. It is held in place by dense connective tissue fibers that are confluent with the gingival fibers. The superior fibers are attached to the gingiva, and the inferior fibers are attached to the bone. Figure 13 shows the dense collagenous nature of the fibers and their attachment to the cementum.

The irradiation produced a marked thickening of most of the arterioles and some of the arteries in both jaws, predominantly in the mandible, while the capillaries and veins appeared normal. The tissue at the base of the crown contained capillaries, and a few arterioles were found in the area, but the vascularity of the tissue was not a prominent feature.

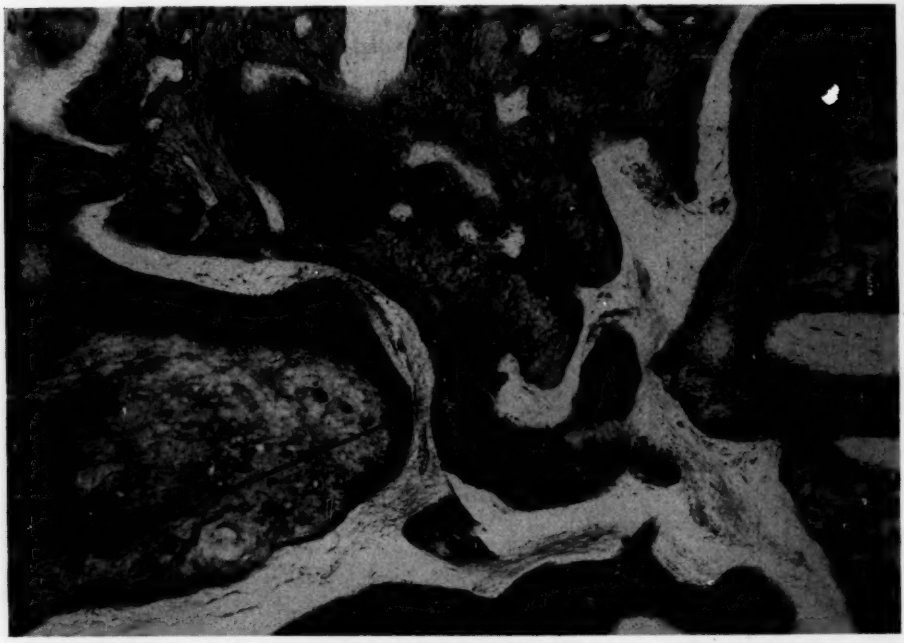


FIG. 10.—Three and one-quarter years post-irradiation, 7500r. Magnification × 155. Ankylosis of a molar tooth. Osteodentin fills the pulp chamber and is continuous with the alveolar bone at the base of the tooth. Note apical foramen with osteodentin formation (*arrow*).

In all teeth examined, there was a layer of osteodentin of variable thickness between the normal dentin and cementum.

DISCUSSION

These observations have important implications for the phenomenon of tooth eruption. From these findings it is apparent, both roentgenographically and microscopically, that pulpal proliferation or root formation was not a factor in the eruption of these teeth. Quite the contrary, the pulp chambers were closed before there was any appreciable migration of the teeth. Because of the lack of root formation, the periodontal ligament was eliminated from consideration as a factor in tooth eruption. The degree of vascularization of the periapical tissues and its relationship to tooth eruption have recently received considerable attention as a prime factor in tooth eruption.[2] In the present experiments, the degree of vascularization of the periapical tissues of the erupting tooth was found to be within normal range. In addition, the main artery and most of the arterioles had a relatively marked degree of irradiation-produced arterio- and arteriolar-sclerosis.

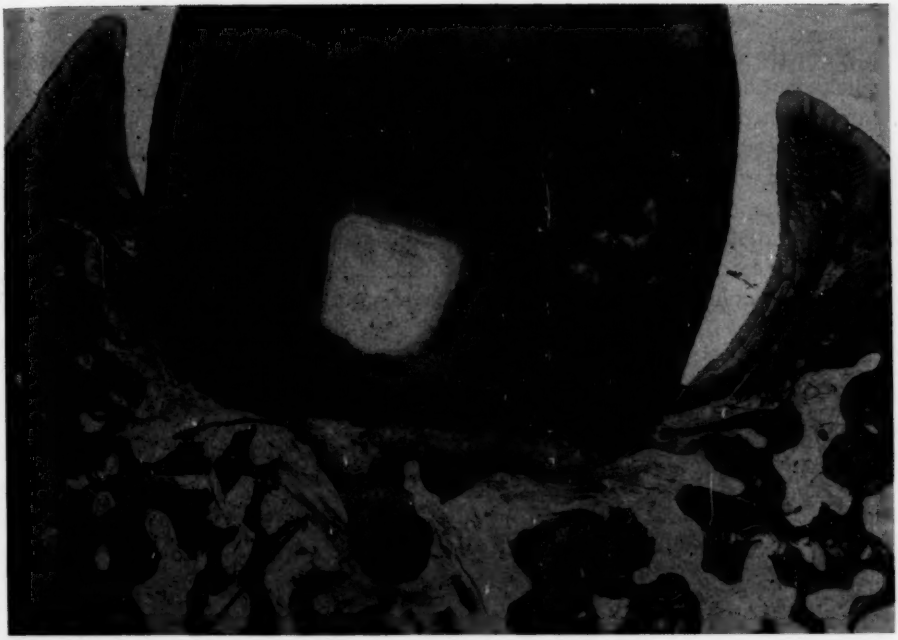


FIG. 11.—Three and one-quarter years post-irradiation, 7500r. Magnification ×30. Fibers of the follicular sac are attached to the alveolar crest and gingiva. The aborted root has one site of differentiated periodontal ligament (*arrow*).

FIG. 12.—Three and three-quarter years post-irradiation, 5500r. Magnification × 30. Fibers of the follicular sac are attached to the alveolar crest and gingiva. No root formation or differentiated periodontal ligament.

When the crown is unerupted, the fibers of the follicular sac are relatively loose, cellular, arranged parallel with the crown, and attached to the cementum. With the crown erupted into the oral cavity, the fibers become much more dense and are oriented more perpendicular to the crown. They are confluent with the gingival fibers of the corium and are attached to the bone of the alveolar crest. These changes demonstrate the growth and maturation of the connective tissue of the follicular sac. This growth and maturation are associated with the occlusal migration of the crown. This is suggestive evidence that the eruption of teeth depends upon the growth of the follicular sac. These findings tend to support the conclusion of O'Brien, Bhaskar, and Brodie.[5]

FIG. 13.—Higher magnification of Fig. 12, showing dense collagenous nature of follicular sac fibers that are confluent with gingival fibers. Fibers attached to normal cementum, above which is osteodentin. Magnification ×270.

Although bone formation at the fundus of the socket was associated with tooth eruption, it could not be determined whether it had an active or passive role in tooth eruption.

The role of the follicular sac and mandibular bone growth in tooth eruption requires further investigation. Experiments aimed at elucidating their function in this phenomenon are now being carried out.

The complete inhibition of further root development by irradiation is a rather well-known phenomenon. The eruption of teeth with short roots so produced has been described in humans.[11, 12] However, the eruption of completely rootless teeth has not been noted. The histologic changes of abnormal dentin formation, pulpal fibrosis, cellular cementum formation, and ankylosis as a result of irradiation have also been reported.[13] The term "osteodentin," while histogenetically inaccurate, is a descriptive

term that has been accepted for the abnormal dentin that is produced by the irradiation-damaged odontoblasts. The complete obliteration of the pulp chamber by osteodentin has not been previously reported.

It could not be definitely determined whether the cessation of root formation is the result of the irradiation effect on the epithelial root sheath or on the odontoblasts. Hansen and English[14] irradiated the heads of rats and stated that the locus of damage was at the proliferating apical end of the developing tooth and involved both ameloblasts and odontoblasts. As previously stated in the present work, the epithelial root sheath was not present in all sections, but, when present, it terminated on the osteodentin. The finding of osteodentin formation suggests that damage to the odontoblast is the primary site for inhibition of further root formation.

SUMMARY

By means of X-irradiation, root formation has been completely inhibited in *Macacus rhesus* monkeys. These teeth erupted into the oral cavity without root formation, pulpal proliferation, or periodontal ligaments. They were held firmly in place by connective tissue fibers of the follicular sac and were in functional occlusion. The eruption of these teeth appears to be dependent upon growth of the folicular sac and/or alveolar bone.

REFERENCES

1. Massler, M., and Schour, I. Studies in Tooth Development: Theories of Eruption, *Am. J. Orthodontics Oral Surg.*, **27**:552, 1941.
2. Bryer, L. W. An Experimental Evaluation of the Physiology of Tooth Eruption, *Internat. D. J.*, **7**:432, 1957.
3. Miller, B. G. Investigations of the Influence of Vascularity and Innervation on Tooth Resorption and Eruption, *J. D. Res.*, **36**:669, 1957.
4. Taylor, A. C., and Butcher, E. O. The Regulation of Eruption Rate in the Incisor Teeth of the White Rat, *J. Exptl. Zool.*, **117**:165, 1951.
5. O'Brien, C., Bhaskar, S. N., and Brodie, A. G. Eruptive Mechanism and Movement in the First Molar of the Rat, *J. D. Res.*, **37**:467, 1958.
6. Engle, M. B. Some Changes in the Connective Tissue Ground Substance Associated with the Eruption of Teeth, *J. D. Res.*, **30**:322, 1951.
7. Baume, L. J., Becks, H., and Evans, H. M. Hormonal Control of Tooth Eruption. I, *J. D. Res.*, **33**:80, 1954.
8. Baume, L. J., Becks, H., Ray, J. C., and Evans, H. M. Hormonal Control of Tooth Eruption. II, *J. D. Res.*, **33**:91, 1954.
9. Baume, L. J. Becks, H., and Evans, Hormonal Control of Tooth Eruption. III, *J. D. Res.*, **33**:104, 1954.
10. Gowgiel, J. M. Experimental Radio-Osteonecrosis of the Jaws, *J. D. Res.*, **39**:176, 1960.
11. Rushton, M. A. Effect of Irradiation on the Dentition, *Am. J. Orthodontics Oral Surg.*, **33**:828, 1947.
12. Stafne, E. E., and Bowing, H. H. The Teeth and Supporting Structures in Patients Treated by Irradiation, *Am. J. Orthodontics Oral Surg.*, **33**:567, 1947.
13. Burstone, M. S. The Effect of X-Ray Irradiations on the Teeth and Supporting Structures of the Mouse, *J. D. Res.*, **29**:220, 1950.
14. Hansen, L. S., and English, J. A. Histologic Changes in the Incisor Teeth of Rats Serially Sacrificed after Receiving 1500r at 200KV X-Ray Irradiation, *J. D. Res.*, **36**:417, 1957.

Pre-eruptive Factors of Tooth Rotation and Axial Inclination

YOUNG H. KIM, F. R. SHIERE, and H. R. FOGELS

Forsyth Dental Infirmary and Department of Oral Pediatrics, Tufts University School of Dental Medicine, Boston, Massachusetts.

Knowledge concerning rotation of permanent teeth is based primarily on posteruptive disturbances. Habitual, mechanical, local, or environmental factors have been cited as etiologic agents. The deviated path of eruption of the permanent incisors, subsequent to traumatic injury or pathologic change, is designated as a pre-eruptive disturbance.[1, 2] Thus the rotation of permanent teeth can be divided into two categories in relation to etiologic factors: (1) rotation of permanent teeth caused by posteruptive disturbances and (2) those caused by pre-eruptive disturbances. If these pre-eruptive disturbances are significant in altering the eruptive path of the permanent incisors, it is possible that similar conditions may influence the eruption of the permanent posterior teeth. There are several adverse factors operative in the pre-eruptive phase of permanent tooth emergence, for example, (1) asymmetrical root resorption of deciduous molars, (2) pathologic involvement of deciduous roots, and (3) the early loss of the first permanent molar prior to the eruption of the adjacent premolars. Logically, these conditions can be differentiated into separate entities as they affect tooth eruption.

According to Morgan's study,[3] it was evident that if a deciduous tooth was retained beyond normal exfoliation time, the succeeding permanent tooth had a tendency to rotate. One reason for this prolonged retention was given as the incomplete resorption of deciduous roots. Another was the lack of co-ordination between the resorption process of deciduous tooth roots and the eruption process of the permanent successors. Morgan's emphasis was focused on prolonged retention as the factor responsible for rotation of the permanent successor.

Ash[2] stated that overretention of a mandibular second deciduous molar may result in a generalized malocclusion. He demonstrated that a minor irregularity of the dentition may cause a generalized dental irregularity. Malocclusions that occur from this type of minor irregularity are common.

The axial inclination of teeth must be considered when occlusion is appraised. The importance of this has been expressed by many workers in evaluating dentofacial balance and harmony.[4–7] These studies were based on axial inclination of anterior teeth, which undoubtedly do influence the profile of the face. However, the axial inclination of posterior teeth has not been fully investigated. It was shown by Marshall-Day[8] and Salzmann[9, 10] that the loss of a permanent molar or molars created abnormal drifting

This study was supported in part by U.S.P.H.S. research grant D-356 from the National Institute of Dental Research, National Institutes of Health.

Received for publication October 21, 1960.

of adjacent teeth and consequently a malocclusion. Strang[11] stated that "the teeth mesial to the space either stand still, . . . may even be tipped distally into the space and an abnormal distal axial inclination of their crowns thus will be established." Such factors have been studied on the basis of posteruptive disturbances. However, the deviation of axial inclination resulting from pre-eruptive disturbances has not been described, as far as the authors are aware. The purpose of this study was to evaluate the conditions that affect rotation and axial inclination of permanent posterior teeth in the pre-eruptive stage.

MATERIALS AND METHODS

Longitudinal records of 503 cases consisting of clinical examinations, full-mouth periapical radiographs, and study models were utilized. The subjects were Caucasian children living in the New England area. From this total, 180 cases of the lower first premolars and 211 cases of the lower second premolars were selected for study. It was determined that lower premolars would provide accurate data, since the periapical radiographs display minimum distortion in these areas.

TABLE 1

TOTAL POPULATION OF LOWER FIRST AND SECOND PREMOLARS BY GROUPS

Group	R4	L4	Total	R5	L5	Total
I.....	47	47	94	47	43	90
II.....	20	15	35	22	20	42
III.....	9	8	17	24	20	44
IV.....	15	19	34	15	20	35

The premolar tooth population was divided into four groups (Table 1). Group I, the normal sample, consisted of 94 first premolars and 90 second premolars. Group II, cases which showed asymmetrical resorptions of deciduous molar roots, consisted of 35 first premolars and 42 second premolars. Group III, cases with pathologic involvement in the apical region of deciduous molars, consisted of 17 first premolars and 44 second premolars. Group IV, cases with loss of the lower first permanent molar prior to the eruption of adjacent premolars, consisted of 34 first premolars and 35 second premolars.

The chronologic age at the time of measurement was obtained for each case. The mean ages and standard deviations for each group are shown in Table 2.

The data concerning sex distribution in each group are presented in Table 3. Although the number of male and female subjects is given, the differences which might have existed in the measurement between the sexes were not analyzed because of the small size of the sample represented in Groups II, III, and IV. However, it is worthwhile to mention that male and female cases were well distributed within each group.

Rotation was expressed by the relationship of the buccolingual cusps of the lower premolars to the lower dental arch contour. The measurements were made on the study models with the aid of a clear plastic-arm protractor. They were read by placing the 90° line of the protractor directly on the centers of the buccal and lingual cusps of

the tooth and then by placing the line of the arm along or parallel to the buccal aspect of the dental arch contour (Fig. 1). When the buccal aspect of the tooth rotated toward the mesial, the measurement was read as a mesial rotation; when rotated toward the distal, it was read as a distal rotation.

In order to determine the mesiodistal or anterioposterior axial inclination of the lower first and second premolars, a direct measurement on the periapical radiograph was made. The measurement of the inclination was obtained from an axial line shown on a radiograph, which runs from the center of the cusp to the apex and an occlusal line

TABLE 2

MEAN CHRONOLOGIC AGES AND STANDARD DEVIATIONS OF LOWER PREMOLARS

GROUP	FIRST PREMOLARS		SECOND PREMOLARS	
	Mean Age (Years)	S.D.	Mean Age (Years)	S.D.
I.....	12.24	0.67	12.23	0.72
II.....	12.07	0.76	12.21	0.73
III.....	11.80	0.91	11.79	1.00
IV.....	12.51	0.77	12.54	0.78

TABLE 3

SEX DISTRIBUTION OF LOWER PREMOLARS

GROUP	FIRST PREMOLARS			SECOND PREMOLARS		
	Male	Female	Total	Male	Female	Total
I.....	50	44	94	49	41	90
II.....	20	15	35	27	15	42
III.....	9	8	17	26	18	44
IV.....	17	17	34	18	17	35

drawn tangential to the cusps of the posterior and canine teeth (Fig. 2). When the coronal portion of the tooth was tipped mesially, it was read as an angle greater than 90°, and, when tipped distally, it was read as an angle less than 90°.

RESULTS

Significant rotations in the erupting lower premolars were observed by using the method previously described (Table 4). In Group I, less than 3° of rotation was found. The severity of rotation increased progressively through Groups II, III, and IV, and rotation of the second premolar was greater than that of the first premolar. According to the *t*-tests, these differences were found to be significant at the 1 per cent level of confidence.

It was observed in Group III (Table 5) that some cases showed pathologic lesions for more than one year and others for a shorter period of time. By subgrouping these

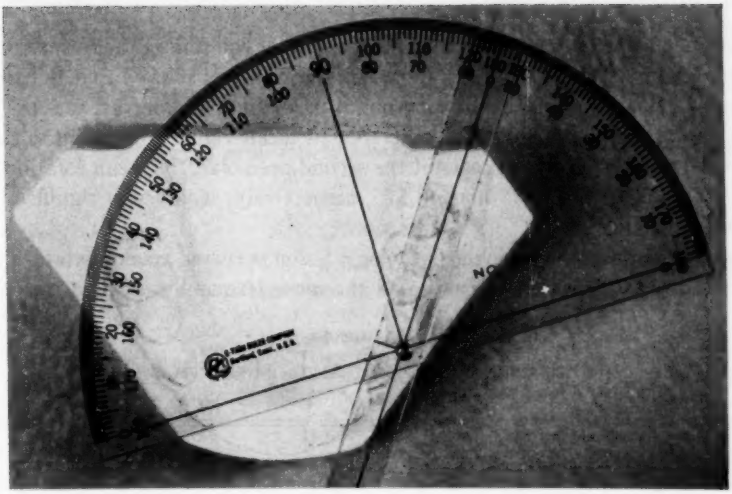


FIG. 1.—Measurement of tooth rotation for the lower left second premolar. The measurement indicates 55° of mesial rotation.

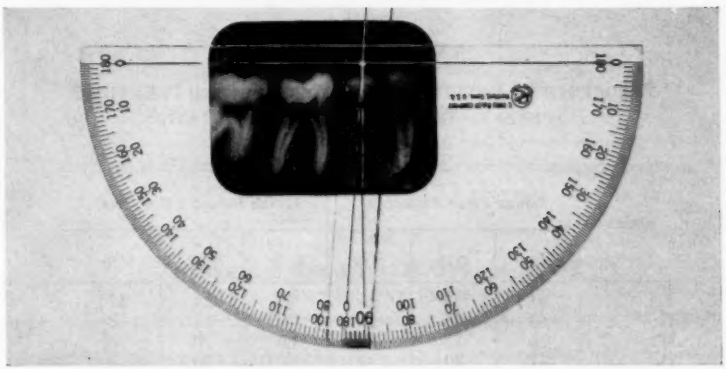


FIG. 2.—Measurement of axial inclination of the lower left second premolar. The measurement indicates 94.5° of axial inclination in relation to the occlusal line.

TABLE 4

DEGREES OF ROTATION OF LOWER FIRST AND SECOND PREMOLARS

GROUP	LOWER FIRST PREMOLARS			LOWER SECOND PREMOLARS		
	Mean	S.D.	*t*	Mean	S.D.	*t*
I.....	2.03°	3.38	.....	2.79°	3.63	.....
II.....	6.26°	5.87	5.615	11.90°	8.13	8.373
III.....	11.71°	7.12	6.993	23.57°	17.71	10.584
IV.....	12.66°	10.21	7.819	25.59°	16.63	11.299

two separate entities, it was found that the severity of rotation in the former (subgroup A) differed from that of the latter (subgroup B). Analysis showed that in cases of the first premolar the mean rotation of subgroup A was 18.17°, in contrast to 8.18° of mean rotation shown in subgroup B. The difference of mean values was found to be significant at the 1 per cent level. In cases of the second premolar, the mean rotations of subgroups A and B were 29.05° and 18.57°, respectively, which was significant at the 5 per cent level.

The approximate age at which a pathologic lesion occurred was determined for each case in Group III. This analysis revealed the most frequent age when this condition

TABLE 5

ROTATION OF FIRST AND SECOND PREMOLARS IN SUBGROUPS OF GROUP III

	Subgroup A			Subgroup B			
	No.	Means	S.D.	No.	Means	S.D.	*t*
First premolars	6	18.17°	4.72	11	8.18°	5.59	3.87
Second premolars	21	29.05°	2.37	23	18.57°	10.17	2.14

TABLE 6*

FREQUENCY OF ROTATION OF FIRST AND SECOND PREMOLARS DIFFERENTIATED BY DIRECTION OF ROTATION (PER CENT)

Group	Lower First Premolars			Lower Second Premolars		
	M/R	D/R	N/R	M/R	D/R	N/R
I	41	8	51	29	24	47
II	63	26	11	79	21	0
III	76	12	12	80	20	0
IV	71	29	0	60	38	2

* M/R = mesial rotation; D/R = distal rotation; N/R = no rotation.

was initiated. The average age was 7.99 years, standard deviation 0.94, for the first premolars, and 8.22 years, standard deviation 1.21, for the second premolars.

Since teeth rotate either mesially or distally, it was necessary to evaluate the frequency of the direction of rotation. This was divided into three categories: mesial, distal, and no rotation (Table 6). According to the data, there was a strong indication that the lower first and second premolars had a characteristic tendency to rotate in a mesial direction.

In order to correlate tooth rotation with eruption age, the supragingival eruption age of each tooth was determined. Mean eruption age and standard deviations were obtained (Table 7). There was no significant correlation between the eruption age and the rotation of erupting premolars.

These data were then compared with those of Hurme.[12] In all groups, the analysis

of the mean eruption ages of the lower first and second premolars corresponds with Hurme's normal range of variation.

In the lower first premolar, Group III demonstrated the earliest eruption time, while, in the lower second premolar, Group II preceded the others in eruption. It is of interest to note the delayed eruption following the loss of the first permanent molar (Group IV).

TABLE 7

COMPARISON BETWEEN MEAN ERUPTION AGES

Group	Mean (Years)	S.D.	Hurme's Mean* (Years)
	Lower First Premolars		
I	10.27	0.82	10.50
II	10.28	0.87	
III	10.00	0.98	
IV	10.69	0.66	
	Lower Second Premolars		
I	10.98	0.85	11.18
II	10.04	0.88	
III	10.80	1.01	
IV	11.16	0.92	

* The mean of boys and girls.

TABLE 8

DEGREES OF AXIAL INCLINATION OF LOWER FIRST AND SECOND PREMOLARS

Group	Lower First Premolars			Lower Second Premolars		
	Mean	S.D.	*t*	Mean	S.D.	*t*
I	88.90°	2.39	.....	92.76°	2.36	.....
II	88.31°	2.32	1.265	93.29°	2.70	0.112
III	88.44°	3.72	0.571	91.74°	4.12	1.711
IV	83.71°	5.19	6.342	88.00°	5.46	6.111

The purpose and the method of analyzing axial inclination of lower premolars was mentioned previously. Data analysis is shown in Table 8.

It was found that the axial inclination of the first premolars was slightly less than a right angle (distal tipping), whereas that of the lower second premolars was slightly greater than a right angle (mesial tipping). Also, there was no significant difference between the means of the normal group and the means of the other groups, with the exception of Group IV. When the mean of this group was compared with the mean of

the normal group, 5° less angulation in both the first and the second premolar cases was demonstrated. This was significant at the 1 per cent level of confidence.

DISCUSSION

Examination of the results reveals that rotation of permanent teeth can occur in the pre-eruptive stage of development. Morgan[3] stated that prolonged retention of primary teeth was one cause of rotation. The present study, in addition, demonstrated that there were other contributing factors, such as asymmetrical root resorption, pathologic involvement of deciduous molars, and the premature loss of the first permanent molar. It was shown in Group II that there was significant rotation of permanent teeth, but that prolonged retention did not appear to be a cause of rotation. However, the retention of the deciduous root tips in Figures 3 and 4 (Case 3) may contribute to the rotation of the second premolar. Group III showed severe tooth rotations,

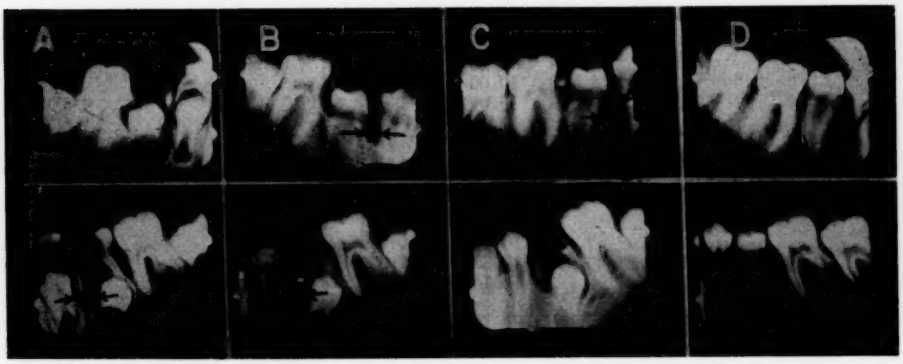


FIG. 3.—Roentgenograms (*upper series—left; lower series—right*) showing radiolucent lesions deviating the eruption pathway of lower second premolars. Case #3 at *A*, 9.1 years; *B*, 10.1 years; *C*, 11.2 years; *D*, 12.2 years.

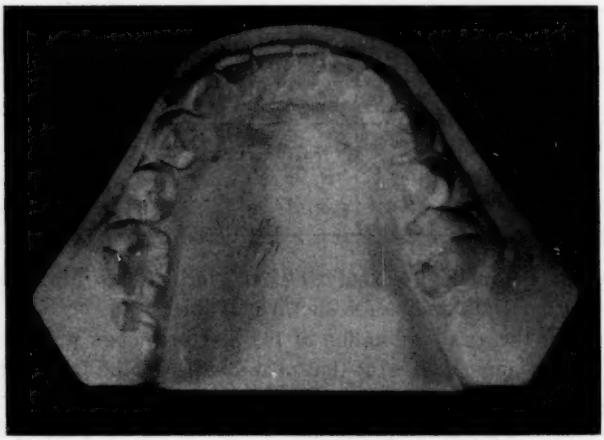


FIG. 4.—Cast taken at 12.2 years showing severity of rotation of second premolars

whereas Group IV demonstrated not only severe rotations but also considerable axial deviation.

Permanent tooth rotations analyzed in Group III may result from pathologic lesions or fluid pressure in the deciduous root area. It is possible that these pressures may be transmitted directly to the dental sac or tooth bud, with the result that the permanent tooth becomes rotated. This response takes place in a direction opposite to the applied force and may alter the pathway of eruption by the interaction of the eruptive forces and the pressure from the pathologic process.

Figures 3 and 5 demonstrate clearly the radiolucent lesions in close approximation to, or in contact with, permanent teeth.

The results of this investigation offer evidence that teeth which cannot be restored to a normal healthy condition should be extracted.

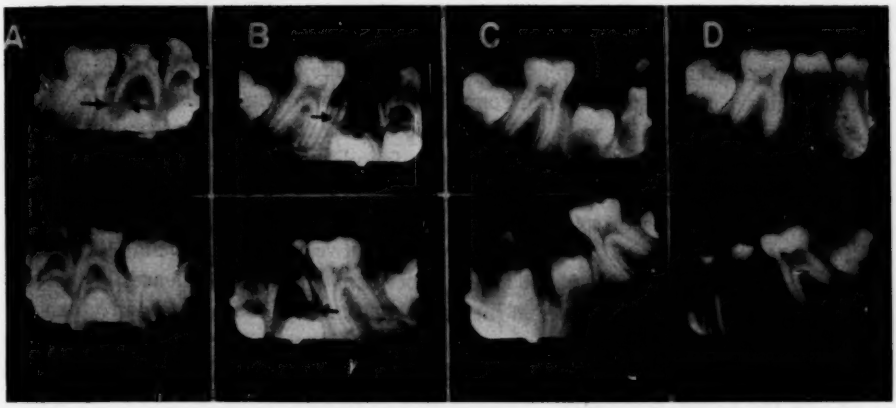


FIG. 5.—Roentgenograms (*upper series—left; lower series—right*) showing increasing severity of rotation of lower left second premolar. Case #144 at *A*, 6.3 years; *B*, 7.3 years; *C*, 8.4 years; *D*, 12.2 years.

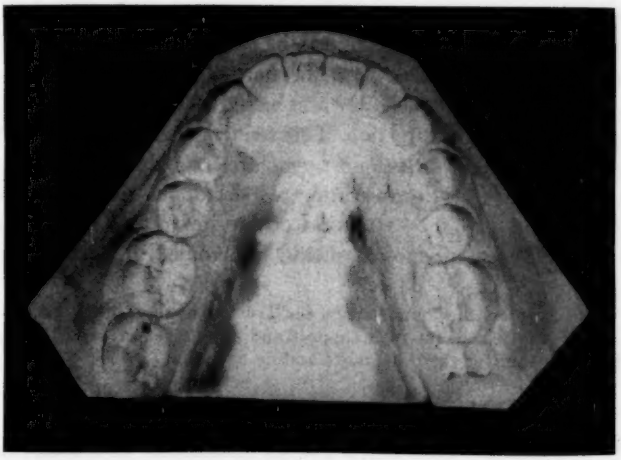


FIG. 6.—Cast taken at 10.4 years showing rotation of second premolar

In conjunction with the loss of the first permanent molar (Group IV), it was found that premolars not only rotate but also incline distally. Therefore, the extraction of the first permanent molar prior to the eruption of the adjacent premolars should be avoided if possible.

SUMMARY

This investigation was undertaken to determine the pre-eruptive causes of rotation and the axial inclinations of the lower premolars. Angular measurements were obtained directly from the study models and radiographs with the aid of an arm protractor. From 503 longitudinal case records, 180 cases of the lower first premolars and 211 cases of the lower second premolars were selected for study.

In Group I, the normal sample, less than 3° of mean rotation was found in both erupting first and second premolars. The mean axial inclinations were 88.90° for the first premolars and 92.76° for the second premolars.

In Group II, the mean rotations were 6.26° for the first premolars and 11.90° for the second premolars. The differences of the means were found to be significant at the 1 per cent level of confidence when compared with the means of the normal sample. The mean axial inclinations were 88.31° and 93.29° for the first and second premolars, respectively, and no significant difference was found between the means of Groups I and II.

In Group III, the mean rotations were 11.71° for the first premolars and 23.57° for the second premolars. The mean differences between this group and the normal sample were found to be significant at the 1 per cent level of confidence. It was also found that cases which had lesions in the deciduous molar root area for more than one year showed significantly greater mean rotations than those which had lesions for less than one year. The means of axial inclination were 88.44° for the first premolars and 91.74° for the second premolars.

In Group IV, the mean rotations were 12.66° and 25.59° for the first and second premolars, respectively. The mean differences between this group and Group I were also found to be significant at the 1 per cent level of confidence. The mean axial inclinations were 83.71° and 88.00° for the first and second premolars, respectively. These mean axial inclinations were found to be significantly different at the 1 per cent level of confidence when compared with the normal group.

In general, it was found that the lower first and second premolars had a characteristic tendency to rotate in a mesial direction. This tendency, as well as the severity of rotation, was found to be greater in second premolars than the first premolars.

It was concluded that (1) the preservation of a deciduous molar with apical pathology is contraindicated, and (2) the extraction of the lower first permanent molar prior to the eruption of adjacent premolars should be avoided, if possible.

The authors wish to express their gratitude to the Trustees of the Abbe Frances Lawton Foundation, Central Falls, Rhode Island, for their support and encouragement, and to Mrs. Edith Tiffany Hill and Mrs. Ruth Oldham for their assistance and co-operation.

REFERENCES

1. Anderson, G. M. *Practical Orthodontics,* p. 204. 8th ed. St. Louis: C. V. Mosby Co., 1955.
2. Ash, A. S. Orthodontic Significance of Anomalies of Tooth Eruption, *Am. J. Orthodont.,* **43**:559, 1957.

3. MORGAN, G. E. Prolonged Retention: When Should Healthy Deciduous Teeth Be Extracted? *J.A.D.A.*, **25**:358, 1938.
4. TWEED, C. The Frankfort Mandibular Plane Angle in Orthodontic Diagnosis, Classification, Treatment Planning and Prognosis, *Am. J. Orthodont. & Oral Surg.*, **32**:180, 1946.
5. MARGOLIS, H. Basic Facial Pattern and Its Application in Clinical Orthodontics, *Am. J. Orthodont. & Oral Surg.*, **33**:631, 1947.
6. DOWNS, W. B. Variations in Facial Relationship: Their Significance in Treatment and Prognosis, *Am. J. Orthodont.*, **34**:812, 1948.
7. GRABER, T. M. New Horizons in Case Analysis—Clinical Cephalometries, *Am. J. Orthodont.*, **38**:603, 1952.
8. MARSHALL-DAY, C. D. Occlusal Abnormalities and Resultant Sequelae Due to High Caries Incidence and Early Loss of the First Permanent Molar, *Internat. J. Orthodont.*, **19**:1269, 1933.
9. SALZMANN, J. A. Variation in Tooth Position Following Extraction of First Molars in Relation to Incidence and Distribution of Dental Caries, *J. D. Res.*, **19**:17, 1940.
10. ———. Effect on Occlusion of Uncontrolled Extraction of First Permanent Molars: Prevention and Treatment, *J.A.D.A.*, **30**:1681, 1943.
11. STRANG, R. H. W., and THOMPSON, W. M. *A Textbook of Orthodontia*, p. 104. 4th ed. Philadelphia: Lea & Febiger, 1958.
12. HURME, V. O. Standards of Variation in the Eruption of the First Six Permanent Teeth, *Child Develop.*, **19**:213, 1948.

The Caries-Conduciveness of a High-Sucrose Diet with and without the Addition of Brewer's Yeast

JOHN HALDI, WINFREY WYNN, and MARY L. LAW
Emory University School of Dentistry, Atlanta, Georgia

Several years ago Dalderup and Jansen[1] reported experiments in which the cariogenicity of a synthetic high-sucrose diet was markedly reduced by the addition of brewer's yeast to the diet. The diet employed in these experiments was that described by Sognnaes.[2] We have fed this diet, which we have designated as the "Harvard diet," in a number of experiments and have found it to be considerably more cariogenic than our Emory diet, although both diets contain the same amount (65 per cent) of sugar.[3] As yeast and liver extract are incorporated in the Emory diet, whereas liver concentrate without yeast is added to the Harvard diet, it was suggested by Dalderup and Jansen that the presence of yeast extract in the Emory diet might explain why this diet is less conducive to dental caries than the Harvard diet.

In later experiments in which this suggestion was submitted to an experimental test, we were unable to obtain a reduction in the caries-conduciveness of the Harvard diet by adding either Fleischmann's yeast or yeast and liver extract. It was therefore concluded that the difference in cariogenicity of the Emory and Harvard diets could not be accounted for by the yeast extract in the Emory diet.

Our failure to obtain results with Fleischmann's yeast comparable to those obtained by Dalderup and Jansen with yeast produced in an Amsterdam brewery suggested to us that the difference in the results of these two experiments may have been due to a difference in the American and European yeasts. In discussing this possibility with Dr. Dalderup, we learned that the brew procedures in America and Europe are completely different and require different types of yeast. We have accordingly pursued our investigation further by obtaining yeast from the same supplier* who provided the yeast used in the experiments of Dalderup and Jansen.

EXPERIMENTAL METHODS

Twenty pairs of littermate albino rats were sialoadenectomized at weaning. From this time on, one animal of each pair was fed the Harvard diet and its littermate the same diet, to which 5 gm. of the European brewer's yeast was added to 95 gm. of the diet. All the animals were allowed to eat ad libitum. The experiment continued for 60 days, and the animals were sacrificed at the conclusion of this period, whereupon the teeth were examined and scored for caries by our usual procedure.[4]

This investigation was supported by Contract DA-49-007-MD-675 with the U.S. Army Medical Research and Development Command, Office of the Surgeon General of the U.S. Army.

Received for publication October 22, 1960.

* The yeast which was provided us by the Povite Production N.V., Amsterdam, was obtained from its subsidary, Amstel Browverez N.V. We wish to express our appreciation to Dr. G. G. A. Mastenbroek for sending us a generous supply of yeast. The name and address of the supplier were kindly sent to us by Dr. Dalderup.

RESULTS

It is apparent, from the results of the experiments shown in Table 1, that the addition of yeast to the diet in these experiments did not reduce either the number or the extent of the carious lesions.

DISCUSSION

The role of yeast added to the diet in modifying the cariogenicity of the diet presents a perplexing problem because of the discrepancies in the results obtained by various investigators. Whereas Dalderup and Jansen[1] found that the addition of 5 per cent brewer's yeast to a high-sucrose diet resulted in a reduction of dental caries, they could not obtain a similar effect by adding yeast to three other diets: one designed to resemble the average Dutch diet as closely as possible, especially with respect to acid-base balance; one which had the actual average composition of the rations of the Dutch

TABLE 1

DENTAL CARIES OF ALBINO RATS FED A HIGH-SUCROSE DIET WITH AND WITHOUT ADDITION OF BREWER'S YEAST

Diet	No. of Animals	Av. No. Carious Lesions	Av. Caries Score (S.E.M.)*	C.R.†
High-sucrose	20	25	61±3.7	1.2
High-sucrose plus 5 per cent yeast	20	24	54±4.7	

* Standard error of the mean.

† The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; 2.0 to 2.9, the difference is of borderline significance; 3.0 or more, the difference is highly significant (J. M. Dunning, *J.D. Res.*, **29**:541, 1950).

population; and another, the coarse corn diet of Hoppert, Webber, and Canniff.[5] Buttner, Cremer, and Hartung[6] were also able to reduce the cariogenicity of a high-sucrose diet by supplementing it with 5 per cent yeast, *Torula utilis*. They obtained similar results with a barley-sugar–fish meal diet.

We find it difficult to explain the difference between the results of our experiments and those of Dalderup and Jansen and those of Buttner, Cremer, and Hartung with a high-sucrose diet as in all these experiments the diets were essentially the same. The discrepancy between the results of our experiments and those of Buttner, Cremer, and Hartung may perhaps be accounted for by the different yeasts used in the two experiments. This, however, was not the case with respect to the experiments of Dalderup and Jansen, since the yeasts used in their experiments and in ours were obtained from the same source. It should be noted that in our experiments the animals were sialoadenectomized, whereas in those of Dalderup and Jansen the salivary glands were not removed. We are inclined to believe, however, that this is not the deciding factor, inasmuch as we have found that, under identical dietary conditions, dental caries develops in our animals whether the salivary glands are removed or left intact. Sialoadenectomy serves only to accelerate the carious process. Furthermore, Buttner, Cremer, and Har-

tung found that yeast was effective in reducing the cariogenicity of the diet fed to sialoadenectomized, as well as to intact, animals.

To add to the confusion of this problem, Buttner and Muhler[7] were unable to obtain a reduction in caries when yeast was added to the same kind of barley-sucrose–fish meal diet as that employed in the experiments of Buttner, Cremer, and Hartung.[6] Subsequently, Buttner and Muhler[8] reported that the addition of 10 per cent irradiated yeast to a stock corn diet actually increased the cariogenicity of the diet. In later experiments the same results were obtained by Muhler and Shafer.[9] These results were the reverse of those obtained in the earlier experiments by Buttner and Muhler with the barley-sucrose–fish meal diet.

Unfortunately, our experiments do not contribute toward a clarification of the perplexing problem of the role that yeast may play in modifying the cariogenicity of diets. Nevertheless, it was deemed advisable to submit our results for publication, in order to bring to the attention of other investigators a few points which have been impressed upon us as a result of our own studies and our review of the literature, namely: (1) in the present state of our knowledge, one is not justified in drawing any general conclusions with respect to the action of yeast on the onset or spread of dental caries; (2) the confusion that now exists on this question indicates that considerably more work is needed to throw light on this problem; (3) future investigations must take into account the possible interrelations of such factors as the composition of the diet, the source and kind of yeast added to the diet, and the presence or absence of the salivary glands in the experimental animals.

SUMMARY

The addition of brewer's yeast obtained from Amsterdam, Holland, did not affect the cariogenicity of our high-sucrose diet. These results were the same as those previously obtained in our laboratories with Fleischmann's yeast. The inconsistencies in the results obtained by various investigators on the modification of the cariogenicity of the diet by the addition of yeast necessitate further investigation of this problem.

REFERENCES

1. Dalderup, L. M., and Jansen, B. C. P. Nutrition and Caries. VII. The Influence of Yeast on Experimental Dental Caries in the Rat, *Intern. Rev. Vitamin Research*, **26**:209, 1955.
2. Sognnaes, R. F. Experimental Rat Caries. I. Production of Rat Caries in the Presence of All Known Nutritional Essentials and in the Absence of Coarse Food Particles and the Impact of Mastication, *J. Nutrition*, **36**:1, 1948.
3. Wynn, W., Haldi, J. Shaw, J. H., and Sognnaes, R. F. Difference in the Caries-producing Effects of Two Purified Diets Containing the Same Amount of Sugar, *J. Nutrition*, **50**:267, 1953.
4. Haldi, J., and Wynn, W. Cariogenicity of Sugar Containing Diets, *J. Nutrition*, **46**:425, 1952.
5. Hoppert, C. A., Webber, P. A., and Canniff, T. L. The Production of Dental Caries in Rats Fed an Adequate Diet, *J. D. Res.*, **12**:161, 1932.
6. Buttner, W., Cremer, H. D., and Hartung, W. Ernährungsfaktoren bei Zahn- und Knochenbildung. VI. Kariesreduktion bei der Ratte durch Zusatz von Trockenhefe (*Torula utilis*), *Deut. zahnärztl. Z.*, **72**:541, 1957.
7. Buttner, W., and Muhler, J. C. The Effect of a Diet Composed of Barley and Fish Meal on Dental Caries in Rats, *J. D. Res.*, **37**:419, 1958.
8. ———. The Effect of Yeast on Dental Caries in the Rat, *ibid.*, **38**:1053, 1959.
9. Muhler, J. C., and Shafer, W. G. The Effect of Irradiated Yeast on Salivary Glands and Dental Caries in the Rat, *J. D. Res.*, **39**:199, 1960.

Dissolution Rates of Enamel and Dentin in Acid Buffers

MICHAEL G. BUONOCORE

Eastman Dental Dispensary, Rochester, New York

It is generally agreed that acid action is a major factor in caries, and lactic acid has been considered primarily responsible. Analyses of plaques, saliva, and carious material, however, have revealed the existence of many acids, any or all of which may contribute to the dissolution of teeth.[1–8] These acids represent a range of chemical properties which may influence the rate and extent of enamel and dentin decalcification, depending on the type of acid or acids involved. Johnston,[9, 10] for instance, has reported that the capacity of organic acids to dissolve synthetic calcium phosphates is considerably influenced by their structure. Because hydrogen ion is common to all acids, variations in solubility effects observed by Johnston can be related to differences in the chemical structure of the anions of the acids.

There is considerable evidence that the structure of anions is extremely important biologically, as, for example, the effect of the anions derived from citric and ethylenediaminetetraacetic acids in increasing the solubility of calcified structures through complex formation. Little attention, however, has been given to the possibility that specific anions may influence the acid dissolution of enamel and dentin. Stralfors[11] has recently shown that buffers of different acids vary in their capacity to dissolve intact enamel surfaces, but he did not attempt to relate these variations to the structure of the acids. Among his buffers, both the anion and the undissociated acid concentration differed, with pH the only common factor. Under such conditions it is difficult to separate effects on solubility which may be due to differences in amount of undissociated acid from those due to differences in the amount or chemical structure of the anions.

In the present investigation, acids were compared for their ability to decalcify enamel and dentin as simple buffers, wherein the pH and anion concentration were equated. Differences among the buffers, therefore, were essentially in the amount of undissociated acid and the kind of anions present. Under these conditions the finding of a definite correlation between undissociated acid concentration and extent of dissolution would be meaningful and suggestive of the absence of specific anion effects. On the other hand, deviations from such a solubility trend line could be indicative of specific anion effects.

MATERIALS AND METHODS

Nine chemically pure, fluoride-free acids were used in this investigation. To eliminate the pH variable, simple buffers of sufficient capacity to resist change in pH during the reaction time were employed. So that the same amount of anions was compared for

This study was supported by U.S.P.H.S. research grant D-225 from the National Institute of Dental Research, National Institutes of Health.

each acid, a given amount of base was titrated with a solution of each acid to pH 4.0. To prepare the buffers, a quantity of standardized sodium hydroxide solution, sufficient to produce a molarity of 0.043 when made up to a final volume of 1000 cc., was added to 850 cc. of distilled water. This solution was then titrated with a solution of known concentration of each acid to pH 4.0, using a meter* to follow the course of the titration. The volume was then brought to the desired level, and the pH rechecked and adjusted if necessary. For proprionic acid an additional buffer was prepared, using half the afore-mentioned amount of sodium hydroxide to give an anion concentration of 0.022 M. This method of preparing the buffers produces solutions having the same pH, acid anion, and sodium ion concentrations, since the total amount of base is completely neutralized in each instance. For other tests in which aspartate and acetate buffers alone were compared, decalcifying solutions of 0.2 M acid concentration were made in the conventional manner by titration of acid with base to pH 4.0. In equating pH and acid anion concentration for the buffers, the concentration of undissociated acid present in each buffer must necessarily be different and directly proportional to the pK of the acid. Except for the anions of the amino acids which are polyvalent, the anions of the other acids are monovalent, and, since the undissociated acid molecules have no charge, their buffers have approximately the same ionic strength. The amino acids, however, exist as zwitterions, and in this paper the natural zwitterionic form is designated as "undissociated acid." The dissociated and undissociated forms of the amino acids are polyvalent and therefore contribute to a higher ionic strength for these buffers. The enamel and dentin powders used were prepared according to the method of Manly and Hodge[12] as modified by Burnett and Battistone.[13]

In one series of tests the dissolving rates of enamel and dentin were determined by mechanically shaking 50-mg. samples with 100 cc. of the buffer solutions for 30 minutes on a wrist-action shaking machine.† In another series, 100-mg. samples were used with 50 cc. of the 0.2 M buffers. In still another series of tests, 50-mg. tissue samples were employed with 100 cc. of buffers which were 0.1 M with respect to the anion concentration. These buffers were used in tests with anorganic enamel prepared according to the method of William and Irvine[14] by taking 200–270-mesh enamel and treating with ethylene diamine to remove organic matter. The resulting enamel has about the same particle-size distribution as the smaller than 270-mesh untreated enamel. The solutions were filtered through selas filtering crucibles of 5-μ maximum pore diameter, and the filtrates were analyzed for calcium and phosphorus. Phosphorus was determined by the method of Quinlan and DeSesa,[15] and calcium was analyzed by the method of Schwarzenback.[16] Both methods were modified as outlined by Koulourides[17] to include the use of perchloric acid to oxidize the organic acids present which interfere in the analysis. At least duplicate runs were made for each buffer, and the runs were repeated when the analytical findings did not check within 5 per cent. All tests were conducted at 25° ± 1° C. Changes in pH were generally within 0.1 of a unit following reaction.

RESULTS

Table 1 shows the results of treating 50 mg. of 120–200-mesh enamel for 30 minutes with 100 cc. of each of the pH 4.0 buffer solutions. Dissolution of enamel was measured

* Beckman Model G, Beckman Instrument Co., Pasadena, California.

† Burrill Corp., Pittsburgh, Pennsylvania.

in milligrams of calcium and phosphate dissolved. Under the heading "Initial undissociated acid molarity," are the concentrations of the buffers with respect to the undissociated acid as determined from titration figures. The amounts of undissociated acid present in the buffers ranged from 0.016 M for formic acid to 0.281 M for propionic acid and bore no definite relationship to the amount of enamel dissolved. This is clearly shown in comparing the low solubility in the aspartic buffer with the high value obtained in the lactate buffer, which had the same concentration of undissociated acid, and is also evident from the result with the glutaric acid buffer, which had about one-third the amount of undissociated acid of the acetate buffer, yet dissolved approximately the same quantity of enamel. Similarly, a comparison of the results with glutaric and glycolic acids leads to the same conclusion. There nevertheless appeared to be a tendency

TABLE 1

SOLUBILITY OF 50-MG. SAMPLES OF 120–200-MESH ENAMEL SHAKEN FOR 30 MINUTES IN 100 CC. OF pH 4 BUFFERS OF SAME ANION CONCENTRATION

(Phosphorus and Calcium Analysis of Filtrates)

BUFFER	INITIAL UNDISSOCIATED ACID MOLARITY	FINAL pH	MG. DISSOLVED		PER CENT DIFFERENCE FROM ASPARTATE
			Ca	P	
Formic	0.016	4.15	10.8	5.0	+ 64.5
Glycolic	0.024	4.11	12.5	5.6	+ 88.0
Aspartic	0.030	4.05	6.6	3.0	.....
Lactic	0.030	4.12	11.3	5.3	+ 72.7
Succinic	0.050	4.04	11.3	5.5	+ 75.3
Glutaric	0.065	4.09	12.3	6.1	+ 92.0
Glutamic	0.086	4.04	8.6	3.9	+ 29.5
Acetic	0.197	4.05	13.5	6.2	+104.2
Propionic	0.281	4.05	12.1	5.9	+ 88.0
Propionic*	0.152	4.05	11.9	5.6	+ 82.1

* 0.022 anion concentration.

for the buffers of the weaker acids to dissolve more enamel than the buffers of the stronger acids. This can be related to the larger amounts of undissociated acid present in the buffers of the weaker acids which provided a greater diffusion potential of undissociated acid from the bulk solution to the surface of the enamel particles, to replenish acid which was used up in the dissolution reaction. The availability of greater numbers of acid molecules for reaction with enamel could be expected to increase the dissolution rate. In line with these considerations are the results with the propionic acid buffers, wherein doubling the anion and free acid resulted in a 6 per cent increase in the amount of enamel dissolved. This result, however, also indicates that large increases in undissociated acid concentration had relatively little effect on dissolution capacity, at least for proprionic acid at the concentration levels used.

The most significant observation in these tests was the unexpected low dissolution demonstrated by the aspartate and glutamate buffers. The large percentage differences in dissolving capacities between the aspartate buffer and the other acid buffers are shown in the last column of Table 1. The comparable dissolving capacities of the aspartate and glutamate buffers are not surprising, in view of the chemical similarity between these two acids.

If the reason for the low solubility of enamel in the aspartate and glutamate buffers is due to factors inherent in the buffers rather than in the enamel, then comparable patterns of dissolution should prevail for dentin, since the dissolving inorganic phase is chemically similar to that in enamel. The results of the solubility tests using 80–120-mesh dentin are shown in Table 2. Contrary to expectations and to their behavior shown with enamel, all buffers showed similar dissolving capacities for dentin. Since the different buffers had the same pH and anion concentration, it would appear that these factors, rather than the kind of anion and/or difference in the amounts of undissociated acid dominated the dissolution of dentin under the experimental conditions.

The difference in solubility between the glutamic acid buffer, which gave the lowest solubility, and the acetic acid buffer, which showed the highest solubility with dentin, was 15 per cent. This difference is small compared with the 104 per cent solubility difference in the enamel series between the aspartate buffer, which gives the lowest solubility, and the acetate buffer, which gives the highest. This indicates that the low enamel solubility in the amino acid buffers was not due to any inherent lack of capacity of these buffers to dissolve apatite. The results of tests to determine whether the difference in solubility behavior of enamel and dentin could be related to the surface areas of these materials are shown in Table 3, along with those for a well-characterized sample of synthetic hydroxyapatite.* Although enamel and dentin solubility increased in both buffers as particle size decreased, the differences in the amounts of enamel dissolved by the aspartate and acetate buffers for each mesh size were approximately the same. Hence increases in enamel surface area, within the limits of the particle sizes used, had relatively little effect on the magnitude of the difference between the dissolving capacities of the aspartate and acetate buffers. Dentin behaved essentially as hydroxyapatite, which showed no difference in solubility in the two buffers.

TABLE 2

SOLUBILITY OF 50-MG. SAMPLES OF 80–120-MESH DENTIN SHAKEN FOR 30 MINUTES IN 100 CC. OF pH 4 BUFFERS OF 0.043 ANION CONCENTRATION

(Phosphorus and Calcium Analysis of Filtrates)

BUFFER	INITIAL UNDISSOCIATED ACID MOLARITY	FINAL pH	MG. DISSOLVED		PER CENT DIFFERENCE FROM ASPARTATE
			Ca	P	
Formic	0.016	4.15	11.4	5.3	−1.4
Glycolic	0.024	4.10	12.0	5.4	+2.4
Aspartic	0.030	4.08	11.5	5.4	.....
Lactic	0.030	4.13	11.9	5.0	0.0
Succinic	0.050	4.05	12.3	5.5	+5.0
Glutaric	0.065	4.08	11.7	5.4	+1.1
Glutamic	0.086	4.03	11.3	5.0	−3.8
Acetic	0.197	4.05	12.4	6.1	−9.3
Propionic	0.281	4.06	11.7	5.7	+1.1
Propionic*	0.152	4.05	11.4	5.3	−1.8

* 0.022 anion concentration.

* Victor Chemical Co., New York, New York.

Table 4 shows a comparison of the solubility of enamel treated with ethylene diamine to increase surface area with intact enamel and dentin in acetate and aspartate buffers, both 0.1 M with respect to anion concentration. The acetic acid buffer at this anion concentration contained an undissociated acid concentration of more than twice that present in the 0.2 M acetic acid buffer (Table 3). With the aspartate buffers, however, there was relatively little difference in undissociated acid concentration. Hence one might expect a tendency toward greater dissolution in the acetate buffer, thus favoring

TABLE 3

DISSOLUTION OF 100 MG. OF ENAMEL, DENTIN, AND SYNTHETIC APATITE BY 50 CC. OF 0.2 M pH 4 ASPARTIC AND ACETIC ACID BUFFERS IN 30 MINUTES
(as Mg. P)

BUFFER	ENAMEL			DENTIN		SYNTHETIC HYDROXYL-APATITE
	120–200 Mesh	200–270 Mesh	<270 Mesh	80–120 Mesh	120–200 Mesh	
1. Aspartate	3.4	4.9	6.5	6.1	8.7	14.8
2. Acetate	7.3	9.4	10.7	7.3	8.7	14.8
Difference 2 over 1	3.9	4.5	4.2	1.2	0	0

TABLE 4

SOLUBILITY OF 50 MG. OF ENAMEL, DENTIN, AND ANORGANIC ENAMEL, EXPRESSED AS MG. PHOSPHORUS IN 100 CC. OF pH 4 BUFFERS OF 0.1 M ANION CONCENTRATION FOR 30 MINUTES

Buffers	Enamel (200–270 Mesh)	Anorganic Enamel (270 Mesh)	Dentin (120–200 Mesh)
1. Aspartic	2.6	6.0	3.5
2. Acetic	6.7	8.8	4.4
Difference 2 over 1	4.1	2.8	0.9

a larger difference between it and the aspartate buffer than that shown in Table 3. This appeared to be the case with 120–200-mesh dentin but not with enamel, where the 4.1-mg. difference in solubility was of the same order as previously found. This 4.1-mg. difference in enamel solubility, measured as phosphate dissolved between the aspartate and acetate buffers, was reduced to 2.8 mg. with anorganic enamel.

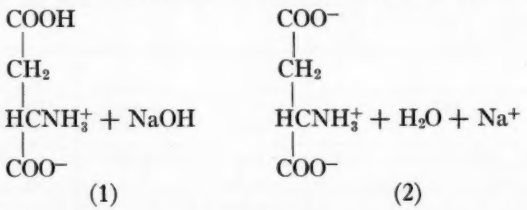
DISCUSSION

The results of this study indicate that differences in the rate of enamel dissolution in buffers at constant pH 4.0 are dependent on the kind of acid used, and not on the anion concentration or to a particular solid/solution ratio (data of Table 1 compared with data of Tables 3 and 4). Dentin, on the other hand, seems to dissolve to about the same ex-

tent in all the buffers. The similarity in behavior patterns of dentin and synthetic apatite, as manifested by the absence of a difference in solubility in acetate and aspartate buffers (Table 3), suggests that the particle size of dentin does not reflect available surface area. Although surface-area measurements were not made on our dentin and enamel powders, they probably would show areas similar to those reported by McCann and Bullock.[18] These authors report surface areas for 325-mesh enamel as being about double those of 200–230-mesh dentin. On this basis it might have been expected that the difference between the amounts of 120–200-mesh dentin dissolved in the acetate and aspartate buffers would be at least as large as the difference obtained by using the smaller than 270-mesh enamel. Hence the absence of a solubility difference with 120–200-mesh dentin, coupled with the similar behavior pattern shown by synthetic apatite, whose large surface area approximates that of bone, suggests that the surface area of dentin as determined by low-temperature techniques is considerably less than that available to a liquid decalcifying medium.

Two basic questions are presented here: (1) Why do the amino acid buffers show a reduced enamel-dissolving capacity compared with the other buffers at the same pH and anion concentration? and (2) Why is this difference in dissolving capacity not demonstrated with dentin?

With regard to the first question, the possibility has been eliminated that the low enamel solubility may have been due to any inherent lack of capacity of the amino acid buffers to dissolve hydroxyapatite. The dissolution observed with dentin leaves little doubt of this. There are, however, certain characteristics of the amino acid buffers different from the others which may help explain their small dissolving capacity for enamel. When the amino acids are neutralized by base to pH 4.0, it is the undissociated carboxyl group that is acted upon, as shown below for aspartic acid:



At the pH 4.0 level, more than half the initial acid 1 (which in this study is called "undissociated acid") has been neutralized to the form 2 (which is termed the "acid anion"). Forms 1 and 2 are in equilibrium with each other. With the non-amino acids the undissociated acid molecules have no charge, whereas the aspartic acid molecule is doubly charged. Moreover, all the anions of the non-amino acids are singly charged, while the anions of aspartic acid have a triple charge. It is apparent, therefore, that, in contrast to the other buffers, the aspartic and glutamic acid buffers contain relatively large numbers of multivalent undissociated acid molecules and anions. Because of the presence of these highly charged ionic species, buffers have a significantly higher ionic strength than the buffers of the other acids.* This factor, however, is not involved in the

* The ionic strength contribution of the highly charged amino acid ions is probably not so great as it would be if the charges were of the same sign. The proximity of positive and negative charges in the same molecule cause partial internal neutralization, which may reduce their net contribution to ionic strength.

reduced ability of the amino acid buffers to dissolve enamel, inasmuch as it would tend to influence the results in the direction of increased dissolution.[19] For instance, it is known that, within certain limits, increases in the ionic strength of a dissolving medium will result in an increased solubility of a sparingly soluble substance. Although these considerations are derived for equilibrium conditions and not specifically for rate studies, the ionic strength factors leading to an increase in equilibrium solubility are operative at all times in the dissolving medium. Hence in the present study the moment-to-moment operation of the ionic strength factors should express itself in an increased enamel dissolution. Evidence to support these considerations has already been reported from our laboratories.[17] The reduced solubility, therefore, found with the amino acid buffers is probably related to the charges of the amino acid ions but independent of their ionic strength contribution.

Two possibilities may be considered to account for the manner in which the high charges might bring about a reduced enamel solubility. These possibilities are based on the fact that the acid dissolution of enamel probably involves the diffusion of undissociated acid molecules to the enamel surfaces from the solution and the diffusion in the opposite direction of reaction products. Let us consider the first possibility. There is evidence that in solutions of the sodium salts of aspartic and glutamic acids large ionic aggregates exist.[20] These ionic aggregates and/or the anions themselves may be attracted to, or adsorbed on the surface of, the enamel crystallites to form a sort of ionic barrier, or surface-bound complexes. An ionic barrier or shell could conceivably be composed of anions or ionic aggregates having their negative charges oriented toward the enamel particle and their positive charges directed toward the solution. The net effect of such an arrangement would be to retard the diffusion of undissociated acid molecules to the apatite surface to provide hydrogen ion for decalcification and, in addition, retard the diffusion of dissolution products to the bulk solution, hence slowing down dissolution rates. The presence of surface-bound complexes would involve, of course, similar considerations to account for the low dissolution rates in the amino acid buffers.[17]

The second possibility is based in part on certain aspects of diffusion as outlined by Sollner.[21] Although the crystallites of enamel are densely packed, there nevertheless exist small spaces between the crystallites which may limit the diffusion of the multicharged undissociated amino acid ions but not the uncharged acid molecules of the other buffers. Because of this, the amount of enamel surface accessible for dissolution in the amino acid buffers may be considerably less than that exposed in the other buffers. The important point here is that, while an increase in external surface area is obtained by reducing the mesh size, even in these smaller enamel particles many spaces exist between the crystallites which may be less accessible to the "undissociated" amino acid ions than to uncharged undissociated acid molecules. It is apparent, however, that, by progressively decreasing particle size, the ratio of accessible to inaccessible surface must increase, which should result in progressively smaller differences between the solubility of the enamel in the amino and non-amino acid buffers. In our tests this tendency was, however, not associated with reductions in mesh size but was evident with enamel treated with ethylene diamine, which apparently increases surface area

considerably more than reduction in particle size from 120–200 to smaller than 270 mesh. The persistence of a comparatively large difference between the solubility of anorganic enamel in the aspartate and acetate buffer (Table 4), however, indicates that the surface available for reaction is nevertheless considerably smaller than that for dentin and synthetic hydroxyapatite.

This brings us to the second basic question regarding why dentin does not show a reduced solubility in the amino acid buffers compared with the other buffers. It is apparent from the results that if charge factors are involved in the reduced solubility with enamel, they are operating differently with dentin. In view of the chemical similarity of the inorganic phases of enamel and dentin, it seems logical to consider that physical differences between these substances may be responsible for their different solubility behavior. One physical characteristic of dentin that is different from enamel is the loose packing and random distribution of considerably smaller crystallites in the former. Consequently, the charged "undissociated" acid ions from the amino acids can diffuse between the crystallites as readily as the uncharged undissociated molecules of the other acids. Solubility rates among the buffers should therefore be similar, other things being equal. Another characteristic of dentin that is different from enamel is the greater amount and kind of organic matter present, which presumably surrounds the apatite crystallites. The existence of this organic matter may prevent, by chemical or physical means, the formation of an ionic shell or surface-bound complexes at the crystallite surfaces to retard dissolution, as postulated for enamel.[17]

The demonstration of specific ion effects as evidenced by the lower solubility rates of enamel in the amino acid buffers compared with the others appears to be a function of both the structural characteristics of the acid ions used and the structural characteristics of the enamel. The finding that certain ionic species can reduce the rate at which an acidic environment dissolves enamel may have particular significance with respect to the initiation and spread of caries in enamel. For instance, it has been previously reported by this author[22] that the addition of aspartic acid to decalcifying buffers, such as lactic, acetic, and pyruvic, reduces the rate of enamel dissolution in these buffers. Moreover, it is interesting to note that the solubility-reducing effect is not related to any rise in the pH of the decalcifying environment. It is conceivable, therefore, that the presence of certain ionic species may have similar effects on the decalcifying rates of the acidic buffer systems of plaques and other areas of caries attack toward enamel. In vitro support for this idea is suggested by the results of Koulourides[19] and Koulourides and Buonocore,[23] which show that anions derived from citric and tartaric acids and lysine, when added to a decalcifying medium, render it less effective in dissolving enamel. In addition, Osborn[24] and more recently Jenkins[25] have reported that certain organic phosphates are capable of similar solubility-reducing effects. Jenkins, in fact, suggests the idea that anticaries action of fluoride may be the result of enolase inhibition in the glycolytic cycle, which may bring about an accumulation of 2-phosphoglyceric acid, which in turn reduces the effectiveness of the lactic acid in dissolving enamel. These investigations, therefore, lend support to our own findings that the decalcifying potential of an acid medium to decalcify enamel can be considerably influenced by the kind of acid or acids present therein.

In contrast to the sensitivity of enamel to the composition of the decalcifying environment, the dissolution of dentin appears to be independent of the type of acid.

Hence, if effects of the sort presented herein occur in vivo, they would be of particular significance with respect to the rate of initiation and spread of caries in enamel but not in dentin.

SUMMARY

A comparison of the rates of solution of enamel and dentin in simple pH 4 buffers of nine acids (acetic, lactic, glycolic, aspartic, glutamic, glutaric, succinic, propionic, formic) indicated that the rate of enamel dissolution was influenced by the kind of acid used. The rate of dentin solubility, on the other hand, which is approximately the same in all the buffers, appears to depend not on the kind of acid but on the pH and anion concentration of the buffer.

The solubility of enamel was lowest in the buffers of the amino acids, with the solubility in the aspartic acid buffer ranging from about 40 to 50 per cent less than in the other buffers.

It has been postulated that the multicharged ions derived from the amino acids, by forming surface-bound complexes with the enamel particles, retard dissolution rates. With dentin the presence of the organic matter may interfere with the formation of surface complexes.

Effects of this sort occurring in vivo could have significant bearing on the rate of initiation and spread of caries in enamel.

REFERENCES

1. Armstrong, W. G., and Atkinson, H. E. Investigation into the Acidic Components of Carious Dentine, *Brit. D. J.*, **95**:8, 1953.
2. Kirch, E. R., Kesel, R. G., O'Donnell, J. F., and Wach, E. C. Amino Acids in Human Saliva, *J. D. Res.*, **26**:297, 1947.
3. Blackwell, R. Q., Fosdick, L. S., and Namajuska, J. Amino Acids in Dental Plaque, *J. D. Res.*, **33**:649, 1954.
4. Miller, B. G., and Muntz, J. A. The Quantity of Lactic Acid in Carious Areas of Human Teeth and Its Relationship to the Soluble Calcium and Phosphate in These Lesions, *J. D. Res.*, **18**:259, 1939.
5. Woldring, M. G. Free Amino Acids of Human Saliva, *J. D. Res.*, **34**:248, 1955.
6. Goldberg, H. V., Gilda, J. E., and Tishkoff, G. H. Paper Partition Chromatography, *J. D. Res.*, **27**:493, 1948.
7. Berry, H. K., and Cain, L. *Quantitative Study of Urinary and Salivary Amino Acids Using Chromatography* (Univ. Texas Publ. No. 5109), 1951.
8. Schulman, E. H., and Robinson, H. B. G. Salivary Citrate Content and Erosion of the Teeth, *J. D. Res.*, **27**:541, 1948.
9. Johnston, H. W. Solubilization of Insoluble Phosphates, *New Zealand J. Sci. and Tech.*, **33**:436, 1951.
10. ———. Solubilization of Insoluble Phophate. II. A Quantitative and Comparative Study of the Action of Selected Aliphatic Acids on Tricalcium Phosphates, *ibid.*, **36**:49, 1954.
11. Stralfors, A. Rate of Dissolution of Dental Enamel, *Trans. Royal Schools of Dentistry, Stockholm and Umea*, No. 1, p. 52, 1958.
12. Manly, R., and Hodge, H. Density and Refractive Index Studies of Dental Hard Tissues, *J. D. Res.*, **24**:103, 1939.
13. Battistone, G. C., and Burnett, G. W. Studies of the Composition of Teeth. III, *J. D. Res.*, **35**:255, 1956.
14. William, J. B., and Irvine, J. W., Jr. Preparation of the Inorganic Matrix of Bone, *Science*, **119**:771, 1954.
15. Quinlan, K. P., and DeSesa, M. A. Spectrophotometric Determination of Phosphorus as Molybdovando Phosphoric Acid, *Anal. Chem.*, **27**:1626, 1955.
16. Schwarzenback. T. *Chimia*, **2**:56, 1948.

17. KOULOURIDES, T. A. The Effect of Organic Anions on Enamel and Dentin Solubility Rates in Acid Buffers. M.S. thesis, University of Rochester, 1958.
18. MCCANN, H. G., and BULLOCK, F. A. Reaction of Fluoride Ion with Powdered Enamel and Dentin, *J. D. Res.*, **34**:59, 1955.
19. GLASSTONE, S. Textbook of Physical Chemistry. New York: D. Van Nostrand Co., 1946.
20. HOSKINS, W. M., RANDALL, M., and SCHMIDT, C. L. A. Aspartic and Glutamic Acids, *J. Biol. Chem.*, **88**:215, 1930.
21. SOLLNER, K. *Electrochemistry in Biology and Medicine,* chap. iii. New York: John Wiley & Sons, Inc., 1955.
22. BUONOCORE, M. G., and SOMMER, B. The Effects of the Addition of Certain Amino Acids to Enamel Decalcifying Buffers, *J.A.D.A.*, **55**:781, 1957.
23. KOULOURIDES, T. A., and BUONOCORE, M. G. Effect of Certain Acid Anions on the Solubility of Enamel and Dentin, *J. D. Res.*, **37**:57, 1958.
24. OSBORN, T. W. B., NORISKIN, S. H., and STAZ, T. Inhibition in Vitro of Decalcification in Teeth, *J. D. Res.*, **16**:545, 1937.
25. JENKINS, G. N. A Note on the Possible Mode of Action of Fluoride in Dental Caries, *Brit. D. J.*, **104**:54, 1958.

Phosphatase in Micro-organisms Cultured from Carious Dentin and Calculus

W. H. BOWEN

Department of Dental Science, Royal College of Surgeons, London

Since the phosphatase theory of ossification was first proposed by Robison,[1] it has been suggested by various authors that a similar mechanism might operate in the mouth for the formation of calculus. There was, however, some controversy as to the source of phosphatase in the mouth. Both Adamson[2] and Smith[3] stated that the gingiva was the source of the enzyme, but this claim was not confirmed by Zander.[4] Glock, Murray, and Pincus, Citron,[6] and Ennever and Warner[7] demonstrated that oral micro-organisms are a source of phosphatase.

In seeking to determine the significance, if any, of microbial phosphatase in calculus formation, an attempt was made to utilize the bacterial flora derived from the contrasting phenomena of caries and calculus formation. Caries involves the removal of calcium salts, whereas calculus entails their deposition. Hence, if microbial phosphatase were connected specifically with calculus formation, its presence could be expected in cultures from calculus but not in cultures from carious dentin, unless different properties were displayed by the enzyme obtained from each source. Alternatively, if similar phosphatase were found in both situations, this would indicate that its existence was not specifically related to calculus deposition, and such a finding would suggest that the influence of the enzyme, if any, might be determined by the local environment.

MATERIALS AND METHODS

Freshly extracted teeth were placed in sterile containers, and samples of calculus and carious dentin were removed within 1–2 hours from the time of extraction. The calculus was removed with a sterile scaler and placed in 1 ml. of sterile 0.85 per cent saline. Using successive sterile excavators, carious dentin from the depth of cavities was removed and placed in saline as described. The dentin and calculus were then crushed, and 0.1 ml. of the resulting suspensions was used to inoculate 250-ml. flasks containing 100 ml. of a culture medium with the following composition: brain-heart infusion,* 18:5 gm.; thioglycollate medium,* 12 gm.; yeast extract,† 1 gm.; distilled water 1,000 ml. The pH of the medium was adjusted to 7.2 with $N/10$ sodium hydroxide, and the medium was sterilized by autoclaving for 15 minutes at 125° C. The flasks were incubated at 37° C. for 48 hours in air.

Following incubation, the resultant growth was harvested by centrifuging at 1,500 *g.* for 10 minutes. It was then resuspended in sterile normal saline and diluted to 0.7

Received for publication November 4, 1960.

* Difco. † Oxoid

optial density in a colorimeter,* set at a wave length of 450 mμ, and adjusted to zero with a blank control of saline, in a $3\frac{1}{8} \times \frac{5}{8}$-inch test tube.

In order to test the validity of using standard optical density as a method for comparing the different cultures in this study, duplicate pour plates were prepared with the fluid medium described, solidified with 1.5 per cent agar, and dilutions of the standardized cell suspensions as inoculum. The plates were incubated for 48 hours in air, and the resultant colonies counted. From this each standardized suspension was found to contain approximately 10^6 cells/ml, and this was uniform over five separate counts from each series.

The substrate used was *p*-nitrophenyl phosphate disodium.† 0.4 ml. of M/100 aqueous solution of the substrate was dispensed into standardized tubes containing 5.6 ml. of a series of M/10 maleic acid buffers ranging in pH from 3.5 to 7.5. In addition to maleic acid buffer, M/10 glycine buffers in the pH range 8–10 and M/10 barbitone buffers in the pH range 7.5–10 were also used. One ml. of the standardized cell suspension was added to the tubes, which were then incubated in a water bath for 1 hour at 37° C. Controls consisted of cell suspension without substrate and substrate in buffer without cells. Following incubation, 3 ml. of N/10 sodium hydroxide was added to each tube to stop the reaction and to develop the yellow color of the *p*-nitrophenol liberated. The test and control tubes were centrifuged at 1,500 *g*. for 10 minutes to precipitate the cells. The optical density of the test solutions was then determined against the buffer-cell suspension control, using a 621 Ilford filter, thus estimating the optical density of the *p*-nitrophenol liberated. The amount of *p*-nitrophenol liberated in substrate-buffer control was negligible.

p-Nitrophenol expressed as μmoles was then determined by extrapolation from a standard curve, constructed by determining the optical density of various concentrations of *p*-nitrophenol in N/10 sodium hydroxide.

Finally, sodium fluoride in appropriate concentrations was added to the test system to determine the effect of fluoride on enzyme activity.

RESULTS

Gram-stained smears of the saline suspensions revealed many Gram-positive and Gram-negative morphologic types, indicating that a mixed flora was cultivated from each source, though no gross difference was observed in smears prepared from cultures of either calculus or carious dentin. However, the cultures of calculus invariably had a much more pungent odor than the cultures from carious dentin.

The phosphatase activity found in the micro-organisms from either source was similar in thirty experiments, both in amount and in pH range; the results of typical experiments are presented. Since *p*-nitrophenyl phosphate disodium is a phosphomonoester, the enzyme is classed as phosphomonoesterase.

Enzyme activity is usually critically dependent on pH. This was observed to be the case in the phosphatase from the micro-organisms in this study. Two peaks of activity were observed, one at pH 5.5, and the other at pH 8.5 (Fig. 1). No single buffer can be used to cover a large pH range; glycine and barbitone buffers were therefore used

* Eel, Model A.

† British Drug Houses.

in the higher pH range, and both gave a peak at pH 8.5. The activity at pH 5.5 was approximately 3.5 times that at pH 8.5, and subsequent studies were performed at pH 5.5.

The activity of the enzyme at pH 5.5 in maleic acid buffer in relation to concentration of substrate was analyzed according to the method of Lineweaver and Burk.[9] Various substrate concentrations were used to compute the velocity as a curvilinear function of substrate concentration (Fig. 2). The results were also used to compute the ratio of substrate to velocity as a function of substrate (Fig. 3). The Michaelis

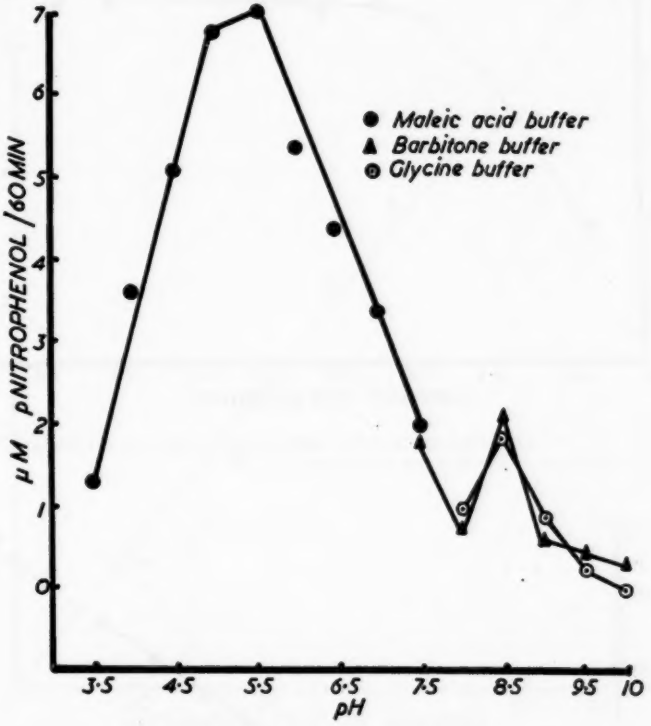


FIG. 1.—Dependence of enzyme activity on pH

constant was calculated and found to be 0.0002 M. The maximal velocity was also calculated in the present study and found to be 11 μmoles per hour. In the interests of economy, 0.4 ml. of M/100 substrate was used in each test and gave 82 per cent of the maximal velocity.

The enzymatic hydrolysis of the substrate was found to be a linear function of time (Fig. 4). Some of the points depart from linearity; this may be due to two reasons, namely, that the enzyme was not saturated or that the enzyme source was relatively crude.

The effect on enzyme activity of using various dilutions of cells is shown in Figure 5. In order to provide an estimate of the number of cells contained in each dilution of the test system, pour plates were prepared, using the most dilute in the series as

inoculum; this dilution was equivalent to one-tenth the original undiluted suspension. The hydrolysis in relation to the relative cell concentration was found to be a linear function. This can be taken as reflecting the enzyme concentrations, as this is the expected relationship.

Sodium fluoride in a concentration of 10^{-6} or 10^{-5} M did not affect the rate of hydrolysis appreciably, but 10^{-4} M caused 30 per cent inhibition and 10^{-1} M 90 per cent inhibition (Fig. 6).

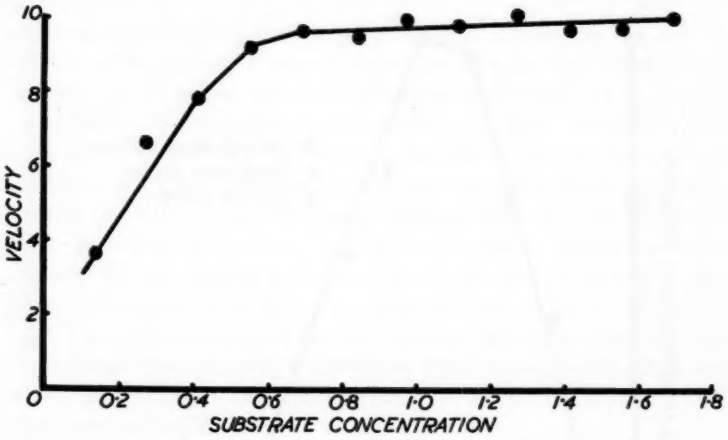


FIG. 2.—The effect of substrate concentration expressed as μ mole, on enzymatic velocity measured as μ moles of p-nitrophenol liberated per 60 minutes.

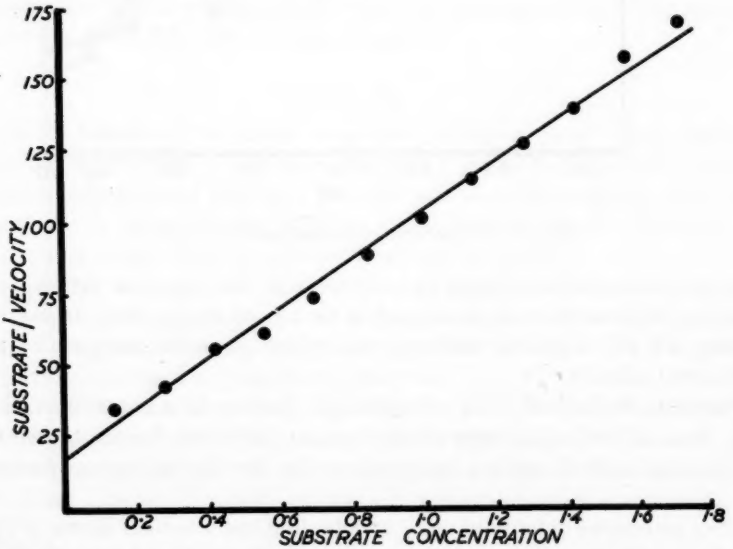


FIG. 3.—Ratio of substrate to velocity as function of substrate. This figure used to calculate maximum velocity and Michaelis constant.

DISCUSSION

The results of the experiments described indicate that the organisms cultivated from calculus and carious dentin have phosphatase activity that is similar in quantity, pH optima, sensitivity to fluoride, and Michaelis constant. The Michaelis constant was found to be 0.0002 M, which is similar to that calculated for phosphatase from *Micrococcus pyogenes*[10] but differs from that of bone phosphatase—the constant for

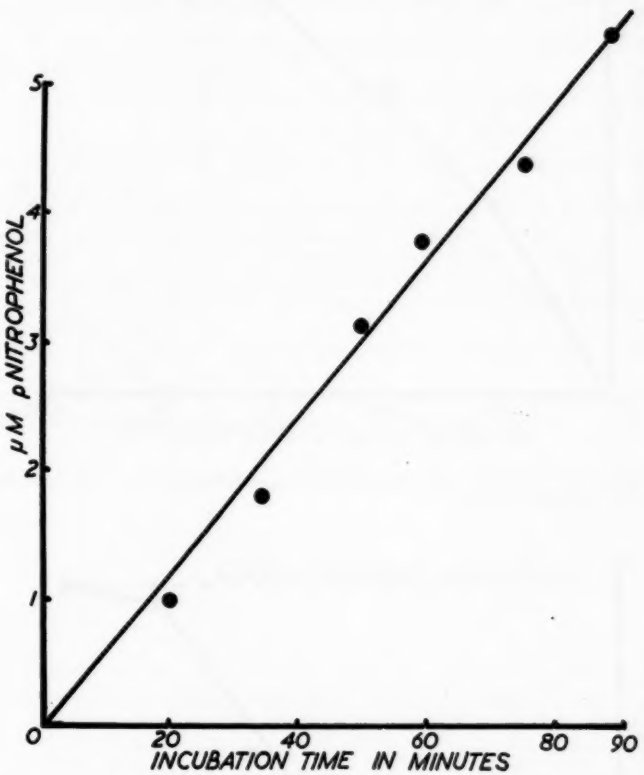


FIG. 4.—The effect of incubation time on enzymatic hydrolysis

which was calculated as 0.003 M.[11] Any suggestion that the presence of bacterial phosphatase can be accorded significance in regard to the deposition of calculus must be weighed against the existence of the similar phosphatase activity found in organisms from carious dentin, where different environmental conditions prevail. It is, of course, possible that phosphatase concerned in calculus formation may differ from the phosphomonoesterase described in this study.

SUMMARY

Micro-organisms cultured from calculus and carious dentin demonstrated a similar phosphatase activity over a wide pH range with peaks at pH 5.5 and 8.5. Sodium

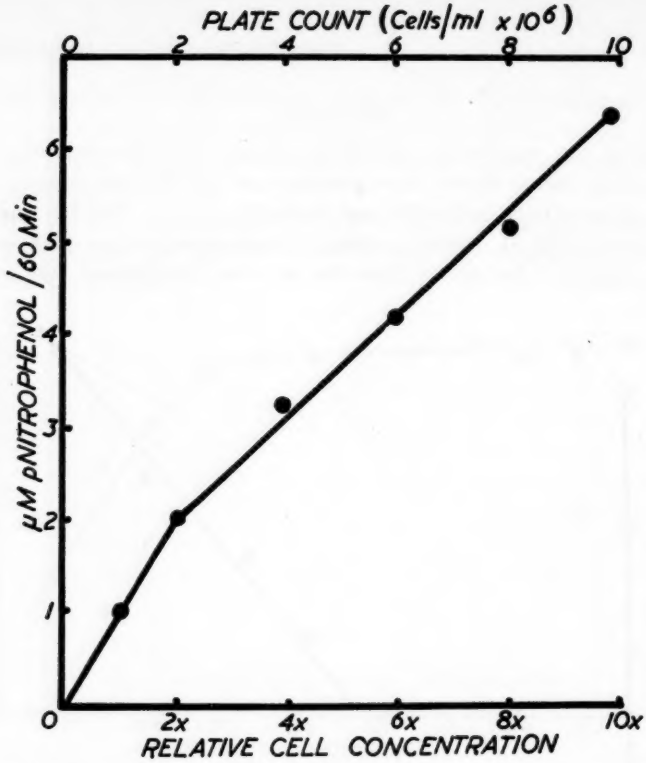


FIG. 5.—The Influence of relative cell concentration on enzyme activity. The most dilute suspension (X) was used to inoculate plates.

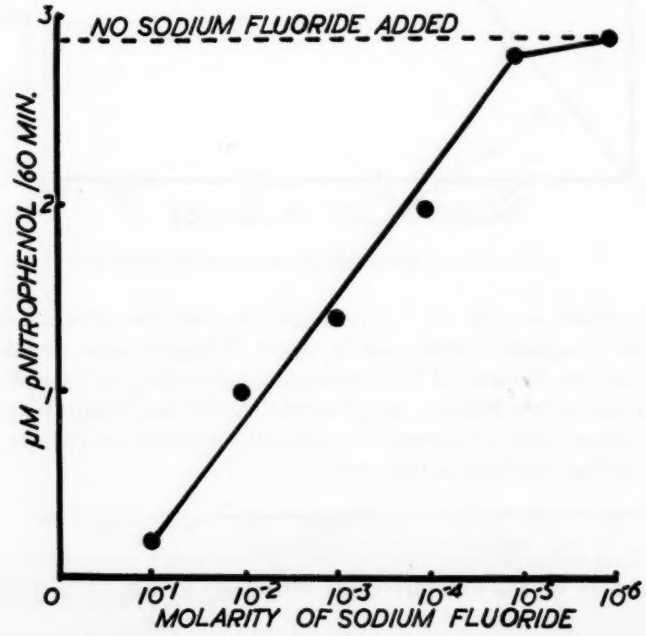


FIG. 6.—The effect of sodium fluoride on enzymatic hydrolysis

fluoride inhibited enzyme activity in concentrations of 10^{-1}–10^{-4} M. Results suggest that microbial phosphomonoesterase is not specifically concerned in calculus formation.

This work was carried out during the tenure of a Quintin Hogg Fellowship in the Department of Dental Science at the Royal College of Surgeons. I am grateful to Mr. E. B. Brain for preparing the illustrations.

REFERENCES

1. Robison, R. The Possible Significance of Hexophosphoric Esters in Ossification, *Biochem. J.*, **17**:286, 1923.
2. Adamson, K. T. The Role of Enzyme Action in the Formation of Dental Calculi, *Australian J. Exptl. Biol. Med. Sci.*, **6**:215, 1929.
3. Smith, G. H. Factors Affecting the Deposition of Dental Calculus, *Australian J. Exptl. Biol. Med. Sci.*, **7**:45, 1930.
4. Zander, H. A. Distribution of Phosphatase in Gingival Tissue, *J. D. Res.*, **20**:347, 1941.
5. Glock, G. E., Murray, M. M., and Pincus, P. The Origin and Significance of Salivary Phosphatase, *Biochem. J.*, **32**:2096, 1938.
6. Citron, S. The Role of *Actinomyces israeli* in Calculus Formation, *J. D. Res.*, **24**:87, 1945.
7. Ennever, J. J., and Warner, B. W. Phosphatase and Oral Actinomyces, *J. D. Res.*, **31**:25, 1952.
8. Sigma Chemical Co. Determination of Serum Acid and Alkaline Phosphatase and Pasteurization of Milk Products Using Sigma 104 Phosphatase Substrate, *Tech. Bull.*, No. 104, 1955.
9. Lineweaver, H., and Burk, D. The Determination of Enzyme Dissociation Constants, *J. Am. Chem. Soc.*, **56**:658, 1934.
10. Barnes, E. H., and Morris, J. F. A Quantitative Study of the Phosphatase Activity of *Micrococcus pyogenes*, *J. Bacteriol.*, **73**:100, 1957.
11. Martland, M., and Robison, R. The Possible Significance of Hexosephosphoric Esters in Ossification. VII. The Bone Phosphatase, *Biochem. J.*, **21**:665, 1927.

Effect of Organic Ions on Solubility of Enamel and Dentin in Acid Buffers

THEODORE A. KOULOURIDES and MICHAEL G. BUONOCORE*
Eastman Dental Dispensary, Rochester, New York

Acid decalcification is generally accepted as a major factor in caries, and it has been usual to place primary responsibility on lactic acid. Analyses of plaques, saliva, and carious material, however, have revealed the existence of acids other than lactic that may be involved in the dissolution of tooth structure.[1–9] These acids, resulting from bacterial fermentation of foods or ingested in the diet, may be present in varying amounts and combinations from one person to another and in a given person from one time to another. At any given acid pH, therefore, plaques, saliva, and carious material may be considered as buffers having hydrogen ion common to a number of anions derived from different organic acids. The capacity of these systems to dissolve tooth structure is the sum of the effects due to various ionic and molecular species present therein. Because the pH of such systems is usually not below 4.5, the concentration of hydrogen ion and undissociated acid must necessarily be small relative to the concentration of the acid anions. Thus, while hydrogen ion and undissociated acid, acting as a hydrogen ion donor, are important in decalcification, the contribution of the anions to this process cannot be ignored.

There are a number of in vitro studies lending support to the idea that specific anions may influence acid decalcification rates. Johnston[10–12] has reported that the solubilizing properties of various organic acids toward calcium phosphates is considerably influenced by their chemical structure. In a semiquantitative study, Osborn, Noriskin, and Staz[13] found that the presence of organic phosphates, such as glycerophosphate, in an acid medium reduces its dissolving capacity for tooth substance. Buonocore and Sommer[14] demonstrated that the addition of aspartic acid to several decalcifying buffers at pH 4.0 resulted in a marked reduction in the amount of enamel dissolved. Jenkins[15] recently indicated that 3-phosphoglyceric acid in an acid medium may reduce its ability to dissolve calcium phosphate.

The above findings suggest that the dissolving capacity of the acidic buffer systems in the mouth, for tooth structure, may depend on the kind or types of acids present therein. This may have direct bearing on the rate of initiation and spread of caries. Since little quantitative work had been done to investigate the effects of different acids on the ability of a buffered decalcifying medium to dissolve enamel and dentin, this research was undertaken.

This study was supported by U.S.P.H.S. research grant D-225 from the National Institute of Dental Research, National Institutes of Health.

Received for publication December 1, 1960.

* Present address: University of Alabama, Dental School, Birmingham, Alabama.

EXPERIMENTAL METHODS

A. *Preparation of enamel and dentin samples and buffer solutions.*—Powdered enamel (120–200 mesh) and dentin (80–120 mesh) used for the solubility tests were prepared according to the method of Manly and Hodge[16] as modified by Battistone and Burnett.[17] Microscope examination showed that the purity of the enamel and dentin powders was above 97 per cent. All acids used were of ACS Reagent grade quality and were fluoride-free.

To determine the effect of the presence of the different acids in a decalcifying buffer on the solubility of enamel and dentin, the following procedure was used. For one series of tests at pH 3.5 and 4.5, the decalcifying acid chosen was formic, which is the simplest acid obtainable that can be buffered effectively for this pH range. The buffer, which will be referred to as the "background buffer," was prepared as follows: A quantity of formic acid sufficient to produce a 0.10 M concentration in 1,000 cc. of solution was weighed, dissolved in 850 cc. of water, titrated with standardized sodium hydroxide to the required pH, and then made up to a liter volume. The pH was then rechecked and corrections made as required. To make the mixed buffer, which consisted of the background acid plus the acid to be tested, the same procedure was followed, with the exception that the test acid was added in sufficient amount to produce a 0.01 M final concentration prior to titration to the required pH.

In additional tests at different pH levels, other acids offering better buffering capacities were used to prepare the background buffers. For instance, for certain tests done at pH 2.6, 0.10 M maleic acid was used, and at pH 5.5 and 6.0, 0.10 M acetic acid was employed. The test acids were added to these background buffers as previously described.

It is apparent that in the titration to the desired pH values, greater amounts of base would be required in the presence of the added acids, particularly with the stronger acids, such as maleic, citric, tricarballylic, and fumaric, where conversion to di- and trivalent anions occurs. The additional sodium and acid anions thus introduced into these systems increased the total ionic strength of some of the mixed buffers considerably above that of the background buffer at the same pH. In addition, since the added test acids are completely neutralized, the total amount of undissociated acid present in the mixed buffers compared with the background buffer was also increased, although certainly by less than 0.01 M in any case. An assessment of the effect of increasing ionic strength and buffer concentration due to the added acid on enamel and dentin solubility has previously been studied and reported by Koulourides.[18] For the buffer systems used in the present study the effect of these factors is not significant.

Essentially, the mixed buffers consist of a relatively large concentration of undissociated background acid and anions derived from it and a considerably smaller concentration of undissociated test acid and anions. Differences between the solubility of enamel and dentin in the background buffer and the solubility in the mixed buffer will be a measure of the effect of the presence of the anions of the test acid on the solubility process. Hereafter, in this report, the term "ion" will be used in place of "anion" to include the dissociation products of certain dibasic amino acids used in this study.

B. *Decalcification procedure.*—The decalcification was carried out by shaking 50 mg. of 120–200-mesh enamel or 68 mg. of 80–120-mesh dentin with 100 cc. of the

various buffers for 15 minutes in 125-cc. capacity flasks* mounted on a wrist-action shaking machine.† Decalcification temperatures were 25° ± 1° C. The above sample weights of enamel and dentin represent nearly equal amounts of calcium plus phosphorus. Following shaking, the contents of the flasks were filtered under vacuum through Selas filtering crucibles of 5 μ maximum pore diameter. The filtrates were analyzed in the majority of cases for both calcium and phosphorus as previously described.[19] In other instances, only one of these was estimated. At least duplicate runs

TABLE 1

EFFECT OF ADDITION OF ORGANIC IONS (0.01 M) TO pH 3.5 FORMATE BUFFER (0.10 M) ON SOLUBILITY RATES OF 50 MG. OF 120–200-MESH ENAMEL (REACTION TIME, 15 MINUTES)

ACIDS	CALCIUM DISSOLVED	PHOSPHORUS DISSOLVED	Ca/P	PER CENT SOLUBILITY CHANGE
	Background Buffer, Formic Acid			
Formic	13.4	6.27	2.14	
Formic	13.4	6.30	2.13	− 0.4
Acetic	13.5	6.30	2.14	+ 0.7
Lactic	12.9	6.35	2.03	− 2.1
Glycine	13.3	6.26	2.12	− 0.5
Mandelic	13.1	6.60	1.99	− 0.2
Malonic	13.5	6.36	2.12	+ 0.9
Fumaric	13.5	6.60	2.05	+ 2.2
Maleic	13.3	6.48	2.05	+ 0.6
Succinic	13.2	6.60	2.00	+ 0.7
Malic	12.0	5.86	2.05	− 9.2
Aspartic	11.1	5.15	2.16	−17.4
Tartaric	10.0	4.90	2.04	−24.2
Glutamic	13.3	6.40	2.08	+ 0.2
Tricarb	12.6	6.25	2.02	− 4.2
Citric	9.6	4.42	2.17	−28.7
Asparagine	13.2	6.43	2.05	− 0.2
Ornithine	12.8	5.78	2.21	− 5.5
Lysine	12.1	5.75	2.10	− 9.3
EDTA	10.6	4.75	2.23	−22.0

were made in all cases, and runs which did not check within 5 per cent were repeated. The pH changes as a result of the decalcifications were never more than 0.10 unit.

RESULTS

Table 1 and Figure 1 show the solubility of enamel measured as calcium and phosphorus found in the filtrates at pH 3.5. Although the ratios of the calcium and phosphorus found in the test solutions varied, they were all sufficiently close to the theoretical Ca/P ratio of enamel to indicate that it was dissolving stoichiometrically. The acids added to the background buffer are arranged generally in order of increasing chain length and substitution by polar groups. In the last column are listed the per-

* Erlenmeyer, Will Corp., Rochester, New York.

† Burrill Corp., Pittsburgh, Pennsylvania.

centage changes in enamel solubility produced by the addition of the different test acids to the formic acid background buffer. In calculating percentage solubility changes, the sums of the calcium and phosphorus values were used when available. It was evident that there was no relationship between solubility reduction and the pK's of the added acids. In going from acetic, the weakest acid, to maleic acid, the strongest, the mixed buffers contained increasing amounts of the ions of the acids added and, conversely, decreasing amounts of undissociated acid.

The addition of the monobasic acids—lactic, acetic, formic, and glycine—did not affect solubility. Acetic acid and glycine have pK's substantially larger than formic and consequently exist largely as undissociated acids in the buffer. The largest solubility reduction was produced by the addition of the tribasic citric acid, followed by tartaric, EDTA, and aspartic. In contrast to aspartic acid, the homologue, glutamic acid, shows no effect, as does asparagine, the amide of aspartic acid.

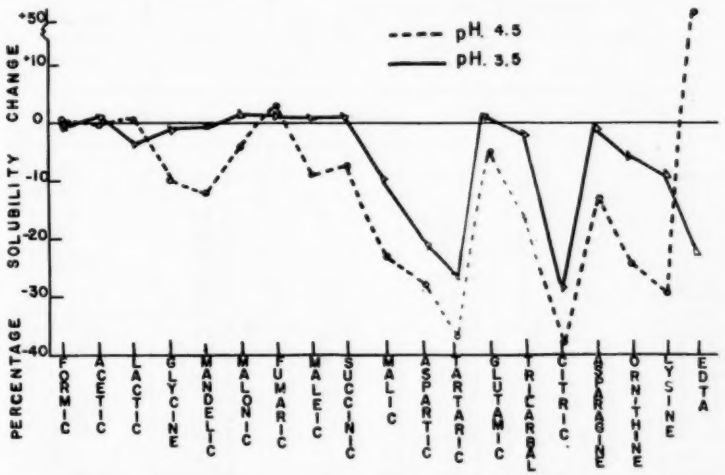


FIG. 1.—Effect of organic ions on the rate of enamel dissolution

The effects of the acids succinic, malic, and tartaric, which are dibasic and contain 4 carbon chains, are interesting. Succinic produced no effect; malic, 9.2 per cent; and tartaric, 24.2 per cent reduction in solubility. In this group of acids, a direct relationship was found between the number of OH groups present in the molecule and the extent of solubility reduction. The importance of the hydroxyl group is also evident when one compares the large reduction shown by citric acid, which contains this group, to the small reduction obtained by tricarballylic acid, which differs from citric only in that the hydroxyl group is replaced by hydrogen.

Lysine and ornithine hydrochloride were used to compared the effects of basic amino acids on solubility with the effects of the acidic amino acids, aspartic and glutamic. It is noteworthy that lysine hydrochloride showed a 9.3 per cent reduction, whereas the reduction with ornithine HCl is 5.5 per cent. In this instance, the amino acid with the longest carbon chain showed the larger reduction, which is the reverse of the situation with aspartic and glutamic.

Table 2 and Figure 1 show the enamel solubility values at pH 4.5. Outstanding is

the solubility shown in the EDTA buffer. In sharp contrast to the reduction observed in this buffer at pH 3.5, the enamel solubility at pH 4.5 with EDTA is increased by almost 50 per cent over that shown by the background buffer. Generally the effects observed at pH 4.5 with enamel are in the same direction, but of greater magnitude, than those observed at pH 3.5. Tartaric, citric, and aspartic at pH 4.5 are again the most potent solubility reducers. The group succinic, malic, and tartaric maintains the same relationship noted at pH 3.5. The fact that aspartic has a stronger effect than malic acid indicates that substituting an amino for a hydroxyl group enhances the solubility

TABLE 2

EFFECT OF ADDITION OF ORGANIC IONS (0.01 M) TO PH 4.5 FORMATE BUFFER (0.10 M) ON SOLUBILITY RATES OF 50 MG. OF 120–200-MESH ENAMEL (REACTION TIME, 15 MINUTES)

ACIDS	CALCIUM DISSOLVED	PHOSPHORUS DISSOLVED	CA/P	PER CENT SOLUBILITY CHANGE
	Background Buffer, Formic Acid			
Formic	4.95	2.41	2.05	.....
Formic	4.97	2.40	2.07	+ 0.1
Acetic	4.95	2.38	2.08	− 0.4
Lactic	4.96	2.43	2.04	+ 0.3
Glycine	4.53	2.10	2.16	− 9.9
Mandelic	4.45	2.06	2.16	−11.5
Malonic	4.70	2.31	2.04	− 4.7
Fumaric	5.12	2.40	2.13	+ 2.2
Maleic	4.14	2.25	1.98	− 9.1
Succinic	4.50	2.28	1.98	− 7.9
Malic	3.82	1.88	2.03	−22.9
Aspartic	3.57	1.73	2.06	−28.0
Tartaric	3.18	1.52	2.09	−36.1
Glutamic	4.70	2.24	2.10	− 5.4
Tricarb	3.98	1.91	2.04	−21.3
Citric	3.06	1.54	1.99	−37.5
Asparagine	4.30	2.10	2.05	−13.0
Ornithine	3.75	1.80	2.07	−24.6
Lysine	3.47	1.78	1.96	−28.6
EDTA	7.47	3.53	2.12	+49.6

reduction. Tricarballylic acid, which is structurally related to critic, produces a rather substantial reduction, whereas pH 3.5 it had only a small reducing effect. The reduction may be related to the presence of larger amounts of ions containing two dissociated carboxyl groups than are present at pH 3.5. The solubility reduction observed with lysine is markedly increased over its effect at pH 3.5. Ornithine, which showed a small effect at pH 3.5, now produces a 24.6 per cent solubility reduction.

Acids which showed little or no effect at pH 3.5 but which demonstrated small reductions at pH 4.5 are glycine, asparagine, glutamic, succinic, mandelic, malonic, and maleic. The monobasic acids, acetic, formic, and lactic, are again essentially without effect. It is interesting that the sterioisomers, fumaric and maleic, show opposite effects, the former increasing solubility and the latter reducing it.

In Table 3 and Figure 2 are shown the effects of a formic acid buffer containing different acid ions on dentin solubility at pH 3.5. The results are presented largely in terms of calcium solubility, as it had been previously established from a number of preliminary results with these acids at pH 3.5 that a measure of the calcium dissolved was a reliable indicator of solubility. Nevertheless, certain randomly selected runs were

TABLE 3

EFFECT OF ADDITION OF ORGANIC IONS (0.01 M) TO pH 3.5 FORMATE BUFFER (0.10 M) ON SOLUBILITY RATES OF 68 MG. OF 80–120-MESH DENTIN (REACTION TIME, 15 MINUTES)

ACIDS	CALCIUM DISSOLVED	PHOSPHORUS DISSOLVED	Ca/P	PER CENT SOLUBILITY CHANGE
	Background Buffer, Formic Acid			
Formic	11.1			
Formic	11.2			− 0.9
Acetic	11.1			0.0
Lactic	11.2			+ 0.9
Glycine	11.6	5.36	2.16	+ 4.5
Mandelic	11.5	5.40	2.14	+ 3.6
Malonic	11.2			+ 0.9
Fumaric	11.8			+ 6.3
Maleic	11.0			− 0.9
Succinic	11.6			+ 4.5
Malic	10.9			− 1.8
Aspartic	10.4			− 6.3
Tartaric	9.8	4.48	2.20	−11.5
Glutamic	11.3			+ 1.8
Tricarb.	11.2			+ 0.9
Citric	9.6			−13.5
Asparagine	11.4			+ 2.7
Ornithine	11.6	5.62	2.07	+ 4.5
Lysine	11.5	5.62	2.04	+ 3.6
EDTA	11.8	5.48	2.16	+ 6.3

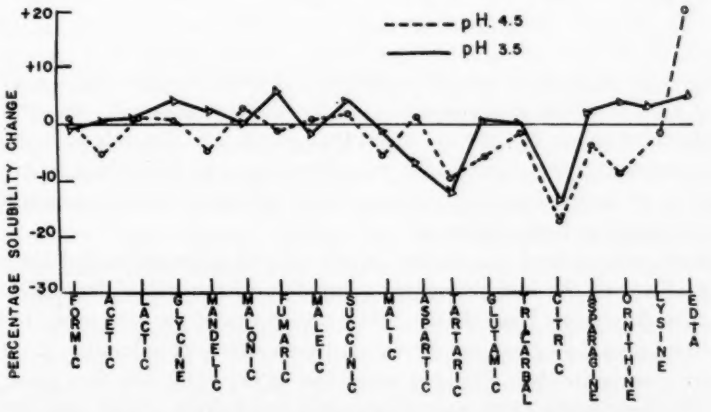


FIG. 2.—Effect of organic ions on the rate of dentin dissolution

analyzed for both calcium and phosphorus. As in other tests, the calcium-phosphorus ratios in these runs indicated stoichiometric dissolution.

The only acids which produced appreciable reductions in solubility were tartaric and citric. Aspartic acid gives only a small reduction in dentin solubility compared with the substantial reduction it gives with enamel. Glutamic acid shows no effect. With a majority of the remaining acids, slight increases in dentin solubility were noted.

Table 4 and Figure 2 show the results of dentin dissolution at pH 4.5. The effects are

TABLE 4

EFFECT OF ADDITION OF ORGANIC IONS (0.01 M) TO PH 4.5 FORMATE BUFFER (0.10 M) ON SOLUBILITY RATES OF 68 MG. OF 80–120-MESH DENTIN (REACTION TIME, 15 MINUTES)

ACIDS	CALCIUM DISSOLVED	PHOSPHORUS DISSOLVED	Ca/P	PER CENT SOLUBILITY CHANGE
	Background Buffer			
Formic	5.95	2.85	.....	.....
Formic	5.95	2.85	2.08	0.0
Acetic	5.72	2.60	2.15	− 5.5
Lactic	6.08	2.81	2.16	+ 1.0
Glycine	6.05	2.75	2.20	0.0
Mandelic	5.67	2.75	2.06	− 4.3
Malonic	6.10	2.96	2.06	+ 3.0
Fumaric	6.00	2.68	2.24	− 1.4
Maleic	5.98	2.88	2.08	+ 0.7
Succinic	6.07	2.90	2.10	+ 1.9
Malic	5.72	2.63	2.17	− 5.1
Aspartic	6.05	2.91	2.08	+ 1.8
Tartaric	5.43	2.51	2.16	− 9.8
Glutamic	5.73	2.62	2.18	− 5.1
Tricarb	5.80	2.88	2.01	− 1.4
Citric	4.96	2.31	2.15	−17.5
Asparagine	5.85	2.71	2.16	− 2.7
Ornithine	5.53	2.59	2.13	− 7.7
Lysine	5.60	2.08	2.09	− 1.4
EDTA	7.28	3.44	2.11	+21.9

essentially in the same direction and magnitude as those found at pH 3.5, with the exception of EDTA, where dentin solubility is increased to about 27 per cent, and with citric acid, which shows a larger reduction than at pH 3.5. Aspartic acid, on the other hand, now produces a slight increase in solubility compared with pH 3.5. On the whole, the solubility of dentin appears to be much less influenced by the composition of the buffer than enamel at both pH levels.

The results with enamel and dentin at pH 3.5 and 4.5 suggest that the solubility-modifying effects of the ions tested are a function of the pH of the buffer medium, which in turn determines their charges. Since the charge of the ions seems to be important in determining the extent and direction of their effects on solubility, it was thought desirable to investigate their effects at lower and higher pH levels than those previously used. The lowest pH (2.6) was chosen only for EDTA, citric, and tricarballylic

acids, since at this pH level the monovalent ion concentrations of citric and tricarballylic and the divalent ions concentration of EDTA are considerably smaller than at pH 3.5. This makes it possible to determine the effect on solubility of the presence of smaller concentrations of the ions of these acids and the associated larger concentrations of undissociated uncharged molecules. For the tests at pH 2.6, a 0.10 M buffer of maleic acid was chosen as the background acid because it forms a good buffer at this pH. In addition, succinic, aspartic, tartaric, and malic acids were also tested at pH 5.5 and 6.0 in acetate buffer to determine whether reversals of behavior such as evidenced by EDTA could be shown for these acids.

Table 5 and Figures 3 and 4 show the effect of the addition of different acids to buffers of maleic (pH 2.6), formic (pH 3.5 and 4.5), and acetic (pH 5.5 and 6.0). The results with the formic acid buffers, which were presented previously in Tables 1 and 3, are also included here, so that the effects over the whole pH range can be compared.

TABLE 5

EFFECT OF ADDITION OF ORGANIC ION (0.01 M) TO DIFFERENT BACKGROUND BUFFERS (0.1 M) AT SEVERAL PH LEVELS ON SOLUBILITY RATES OF 50 MG. OF 200–MESH ENAMEL (REACTION TIME 15 MINUTES)

Acids Added	Background Buffers														
	Maleic, pH 2.6			Formic, pH 3.5			Formic, pH 4.5			Acetic, pH 5.5			Acetic, pH 6.0		
	Ca (Mg. Diss.)	P (Mg. Diss.)	% Sol. Change	Ca (Mg. Diss.)	P (Mg. Diss.)	% Sol. Change	Ca (Mg. Diss.)	P (Mg. Diss.)	% Sol. Change	Ca (Mg. Diss.)	P (Mg. Diss.)	% Sol. Change	Ca (Mg. Diss.)	P (Mg. Diss.)	% Sol. Change
Control	16.1	7.75	.....	13.4	6.27	.....	4.95	2.41	.....	1.42	0.67	.....	0.70	0.33	.....
Tricarb	16.2	7.80	+ 0.6	12.6	6.25	− 4.2	3.88	1.91	−21.3	1.10	0.52	−22.5	0.61	0.27	−14.5
Citric	15.8	7.60	− 1.9	9.6	4.42	−28.7	3.06	1.54	−37.5	1.85	0.80	+26.8	0.97	0.46	+38.7
Succinic	.....	.....	.....	13.2	6.60	+ 0.7	4.50	2.28	− 7.9	1.35	0.65	− 4.3	.....	.....	.....
Malic	.....	.....	.....	12.0	5.86	− 9.2	3.82	1.88	−22.5	1.17	0.55	−17.7	0.59	0.30	−13.6
Tartaric	.....	.....	.....	10.0	4.90	−24.2	3.18	1.52	−36.0	1.10	0.53	−22.0	.....	.....	.....
EDTA	13.1	6.38	−18.4	10.6	4.75	−22.0	7.47	3.53	+49.4	5.80	2.61	+300.0	.....	.....	.....
Aspartic	.....	.....	.....	11.1	5.15	−17.4	3.57	1.73	−28.0	0.99	0.50	−28.7	0.65	0.31	− 6.8

It is important not to make strict comparisons with respect to the magnitude of the effects obtained in different buffers. For instance, the addition of some acids to formate and acetate buffers at pH 4.5 may not affect solubility to the same extent. That this is so is shown in Table 6. In this table the effect of the addition of citric and tricarballylic acids to formate and acetate buffers reveals that, although the effects are in the same direction and relative order, they nevertheless differ in magnitude. On the basis of exploratory work, not reported herein, it was found that with other background buffers, such as lactate, patterns similar to those observed in acetate and formate buffers were obtained. Therefore, in commenting on Table 5, comparisons of effects at different pH levels will be made only when the same background acid is used. In graphing the results of Table 5 (Figs. 3 and 4), however, the solubility values obtained in buffers of different acids have, nevertheless, been linked. If the foregoing comments are kept in mind, misinterpretations of the graph will be avoided.

To bring out the influence of structure on the effects observed, the acids have been arranged according to groups in Table 5: (1) citric and tricarballylic, (2) succinic, malic, and tartaric, (3) aspartic and EDTA. It is noticed that, at pH 2.6, citric acid, like tricarballylic, loses its capacity to reduce enamel solubility. At pH 5.5, citric acid

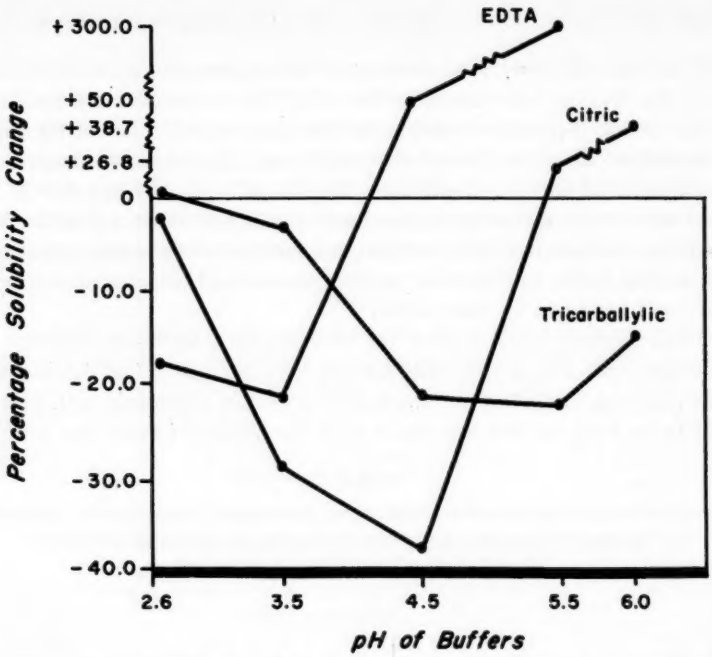


FIG. 3.—Influence of pH on the effects of organic ions on enamel dissolution rates

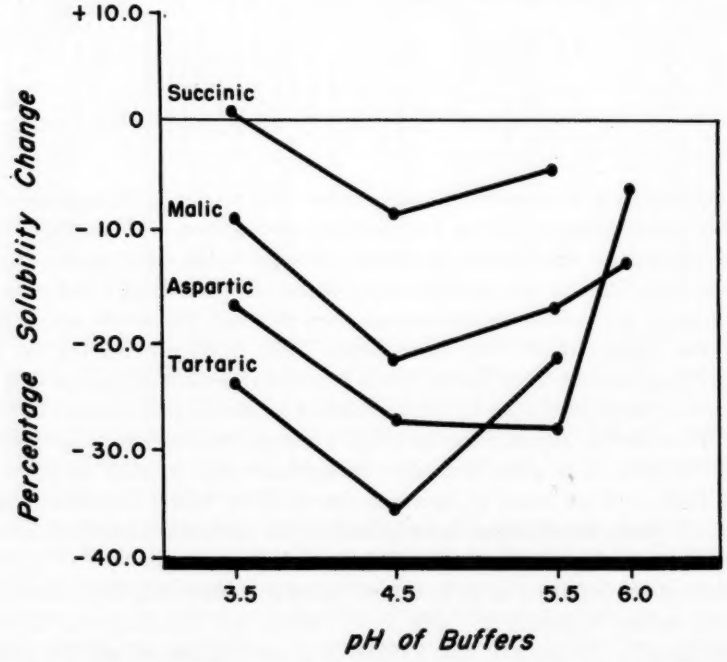


FIG. 4.—Influence of pH on the effects of organic ions on enamel dissolution rates

increases the solubility of enamel, completely reversing the effect it shows at pH 4.5. At pH 6.0, the solubility-enhancing effect is more pronounced (38.7 per cent). The enhancing effect is probably due to complexing of calcium in solution by citrate ions. The graph of tricarballylic solubility appears to reverse its direction between pH 5.5 and 6.0, although it is still reducing enamel solubility (14.5 per cent) even at the latter pH. On the basis of the results at pH 6.0, it is interesting to note that the substitution of citric for tricarballylic in an acetate buffer would result in a 53 per cent increase in the enamel solubility rate. The possibility that precipitation of an insoluble calcium tricarballylate might be responsible for the reduction observed was discounted for two reasons. The first is that the Ca/P ratios of the filtrates were within the theoretical range for enamel. The second is that tests indicated that calcium tricarballylate does

TABLE 6

COMPARISON OF EFFECT OF ORGANIC ION ADDITION TO PH 4.5 FORMATE AND ACETATE BUFFERS ON SOLUBILITY OF 50 MG. OF 200-MESH ENAMEL (REACTION TIME, 15 MINUTES)

Acids Added	pH 4.5, Background Buffers					
	Acetic			Formic		
	Ca	P	% Sol. Change	Ca	P	% Sol. Change
Control.....	4.83	2.42	.....	4.95	2.41	.....
Tricarb.....	4.03	2.01	−16.6	3.88	1.91	−21.3
Citric.....	3.69	1.75	−25.0	3.06	1.54	−37.5

not precipitate at pH 6.0, even at concentrations of acid ten times that used in the tests.

The results with succinic, malic, and tartaric at pH 5.5 substantiate the relationship observed among these acids at pH 3.5 and 4.5. Again, the progressive introduction of hydroxy groups to succinic produces ions showing greater solubility-reducing effects. The precipitation of insoluble calcium salts of succinic, malic, and tartaric acid at pH 5.5, to give low calcium values, was discounted for the reasons presented for tricarballylic acid.

At pH 6.0 the results for EDTA, succinic, and tartaric acids are not presented because, with EDTA, further increases were to be expected and, with succinic and tartaric acids, difficulty in getting consistent results was experienced. Between pH 5.5 and 6.0, both malic and aspartic acids show a change in the direction of their effect on enamel solubility, although both continue to reduce solubility. Their smaller solubility reductions at pH 6.0, compared with pH 5.5, may indicate a change in the balance between ion types which increase solubility and others which reduce it. At pH 6.0, malic and tricarballylic acids have similar effects. This suggests that the presence of anions containing three dissociated carboxyl groups are equivalent in effect to ions having only two such groups and a hydroxy group.

DISCUSSION

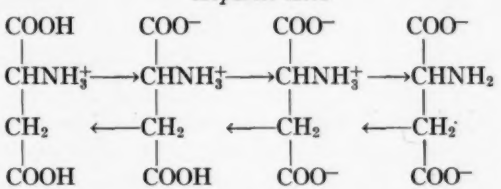
The addition of different acid ions to decalcifying buffers can both increase and decrease the solubility of enamel and dentin. Moreover, depending on the pH, the addition of the same acid can cause opposite effects. The small increases in solubility observed could be expected as a result of the increased ionic strength and/or buffer concentrations of the mixed buffer due to the added acid. The observed greater sensitivity of dentin compared with enamel in this regard is consistent with previous data of Koulourides.[18] The large increases, such as evidenced by EDTA and citric acid at certain pH levels, cannot be accounted for on the same basis. The large solubility increases are probably due to certain complexing abilities of the acid ions. Complexing in solution is, of course, capable of increasing the solubility of difficult soluble salts by combining with its cations to form soluble, relatively undissociated complexes, thus effectively removing cations from solutions to reduce common ion effects which tend to retard dissolution rates.[20] That EDTA produces its solubility increase at a lower pH than citric indicates that it is converted sooner to an ion form which is able to complex calcium in solution. This increase is probably the result of the complexing of calcium in solution by trivalent ions of EDTA which exist at this pH but which are virtually absent at pH 3.5, where reduction in solubility was observed. The marked solubility-increasing effect of EDTA as pH rises is associated with increases in the numbers of ions able to form soluble calcium complexes. That the presence of the hydroxy group in citric acid is important for such complexing is evidenced by the fact that tricarballylic acid, which lacks this group, does not increase solubility even at pH 6.0. Although the solubility increases observed are interesting, the reductions observed in this study have greater interest, since, as far as we know, general effects of this sort have not been previously reported.

The reductions in solubility appear to be related to structural characteristics of the ions of the acids used. The presence of the ions of the monocarboxylic acids, such as acetate and formate, having no polar groups other than carboxyl in their structure, do not produce reductions. The ions of the dicarboxylic acids—maleic, malonic, and succinic—having no other polar groups show only small effects, and these only at the higher pH levels used in the tests. The ions of tricarboxylic acids produce substantial reductions except at pH 2.6, where no reductions are evident. Since our titration data show that, at pH 2.6, roughly 25 per cent of the citric and tricarballylic acids are converted to the monovalent form, it may be inferred that the presence of the monovalent ions with or without a hydroxyl group do not significantly alter the dissolution pattern of the background buffer. Some clarification is needed, however, of the finding that in the presence of monovalent citrate ions there is little solubility reduction at pH 2.6 but a rather substantial effect at pH 3.5. In explanation, two factors can be considered. One is that at pH 2.6 the acid attack is so strong that any tendency for the citrate ions to repress solubility is overwhelmed. The other is that at pH 2.6 there are considerably smaller amounts of the ions than at pH 3.5 and that they are present in a decidedly stronger acid medium. Consequently, acid ion effects tending to reduce solubility may be masked at pH 2.6 but not at pH 3.5. The importance of the hydroxyl group in obtaining reductions is shown with the group succinic, malic, and tartaric, where the extent of reduction is directly related to the number of OH groups present in the ions.

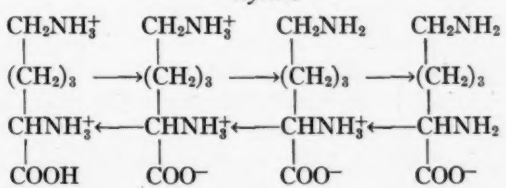
From the results at pH 3.5 it appears that, with the exception of lysine, acids producing substantial reductions exist principally as monovalent ions having both hydroxyl and undissociated carboxyl groups in their structure or are present in large proportions as ions having two dissociated carboxyl groups and an amino group—for instance, EDTA and aspartate ions. The increased solubility reductions observed at pH 4.5, as compared with pH 3.5, are associated with either the appearance of, or an increase in the amount of, such ions in the buffers. The exceptions are mandelic, which is monobasic, and succinic, which is essentially monovalent at pH 4.5. Again, the fact must be considered that the specific anion effects tending to reduce solubility are manifest to a greater extent at pH 4.5 because the acid attack is much weaker than at pH 3.5. That divalent ions with polar groups have the strongest capacity to repress dissolution is supported by the significant reduction observed with EDTA, which exists in part as divalent ion at pH 2.6.

The reductions observed with lysine and ornithine, however, are produced by polyvalent ions of different net charge than the others. At pH 3.5, lysine and ornithine each exist in solutions predominantly as mixtures of ions of two forms, one in greater amount, having two positively charged amino groups and one negatively charged carboxyl group, the other having two positively charged amino groups and an undissociated carboxyl group. The former is, therefore, a trivalent ion of a net positive charge of 1; the latter may be considered an undissociated acid having a double positive charge. The trivalent ions of these acids differ from those of aspartic and glutamic in that the latter have a net negative charge of 1. The dissociation of these acids as pH increases is shown below. The dissociation of glutamic acid and ornithine proceeds in

Aspartic Acid



Lysine



a manner similar to that of aspartic acid and lysine, respectively. The larger reductions obtained at pH 4.5 may be related both to the presence of larger amounts of the triply charged ions than at pH 3.5 and to the less intense acid attack. The fact that marked reductions of the same magnitude are obtained with trivalent ions, such as aspartate, having a net unit negative charge and ions from lysine and ornithine having a net unit positive charge suggests that the nature of the charge may not be as important as the presence of multiple charges on the ions.

With respect to glutamic acid, the lack of solubility reduction is rather surprising in view of the strong reducing effect observed with aspartic acid. These acids are similar in strength and structure, differing only by an additional CH_2 group between the amino and the secondary carboxyl group in glutamic acid. Their solutions consist of divalent ions, with the secondary carboxyl undissociated and trivalent ions representing the form in which the second carboxyl is dissociated. The proportion of each ionic species in both buffers is of the same order. It is difficult to explain the marked difference in the solubility-reducing effects of aspartic and glutamic acids unless one considers that the longer carbon chain length in the glutamate ion may prevent steriochemical effects obtainable with aspartate ion which retard the dissolution of enamel. It is noteworthy that the longer carbon chain length of glutamic acid has been considered responsible for the fact that it is a weaker calcium chelator than aspartic acid at alkaline levels.[21] In addition, some workers have noted that the conversion of one of the carboxyl groups of aspartic and glutamic acids to the amide produces a molecule showing weaker chelating properties than the parent substance.[22] It is evident from the lack of effect of asparagine on solubility that the effect observed with aspartic acid is related to the presence of two dissociated carboxyl groups.

Another structural consideration is revealed in comparing the effects of maleic and fumaric acids, where only the cis form produced a reduction in solubility.

In general, concerning structural characteristics, it may be said that the capacity to reduce solubility at certain pH levels is shown by molecules having structural elements favorable for the complexing of calcium in solution at other pH levels to cause an increase in dissolution. The pH at which they reach this latter stage is related to the pK's of the carboxyl groups involved in this complexing. The persistence of the reducing effect of aspartic, tricarballylic, and malic up to pH 6.0 suggests that the ions are not yet converted to a form suitable for complexing calcium in solution. For instance, with aspartic acid, this might occur at a pH where the positively charged amino group lost its proton.

The differences in solubility behavior of enamel and dentin may be related to the type of surface that each presents to the decalcifying medium. The possibility has previously been suggested that the ions which demonstrate reduction with enamel may, by reason of polar groups, molecular size, charge, or a combination of these factors, concentrate at or near the surface of the enamel particles, forming a sort of ionic shell or perhaps surface-bound complexes which serve to retard the action of hydrogen ion and additionally retard the diffusion of dissolution products to the surrounding bulk solution.[18] Evidence for the firm binding of a citrate complex to apatite crystals of bone under acid conditions has recently been reported by Taylor.[23]

The absence of a reduction with dentin may be related to its organic matter, which completely surrounds the apatite crystallites and, by its presence, may retard the diffusion of certain ions in the buffer to the crystallite surfaces. It may be postulated, therefore, that certain ions, because of large charge or size, are less able to diffuse than others. In fact, some ions may be completely blocked, while others are only partially so; consequently, their "protective action" is entirely absent or considerably reduced. The contrasting effects on dentin and enamel observed with EDTA and lysine may be related to the fact that their ions are polyvalent, whereas the ions of citric and tartaric, which show a solubility-reducing effect with tooth tissues, are monovalent. On this

basis, the demonstration of a reduction in dentin solubility with citric and tartaric acids may be explained by postulating that their ions are able to diffuse more readily than the ions of EDTA and lysine, which are larger and multicharged. The smaller reduction in dentin solubility as compared with enamel produced by the addition of citrate and tartrate to the formate buffer may be considered as the difference between the acid ion factors tending to repress dissolution and the stronger solubility-enhancing effect that increased buffer concentration and ionic strength have on dentin. This same explanation may also account for the observation that lysine addition slightly increases the solubility of dentin, whereas with enamel, a strong opposite effect is noted.

The increases and reductions in solubility reported indicate that acid action is influenced by factors which must be considered specific functions of ions present in the decalcifying medium. This fact may have some broad significance in certain biologic phenomena, such as the resorption and deposition of calcified structures which may be influenced by the kind of specific anion effects herein presented.

The results reported have bearing on theoretical considerations of dental caries which were the starting point of this study. They suggest that the kinds of acids present in plaques and areas of caries attack may influence the capacity to decalcify enamel and, to a limited extent, dentin and that the strength of the caries attack may be modified by the kind of acids found in the mouth, as well as by their quantity. There is increasing support for the idea that acid action in dental caries occurs intermittently as repeated short periods of decalcification. Since our experiments involve short-term reaction rates, the effects observed can be more validly related to oral conditions than if they were equilibrium effects. Further understanding of this subject might help to explain the lack of correlation between caries incidence and such factors as salivary and plaque pH, buffering capacity, and total acidity.[24–30]

SUMMARY

The effect of the addition of nineteen different acids to decalcifying buffers on the solubility rates of enamel and dentin over the pH range 2.6–6.0 was determined. The experimental approach used permitted an evaluation of the specific effects of the characteristic ions of the acids. The ions evaluated were derived from naturally occurring and biologically important organic acids and certain synthetic substances, such as ethylenediaminetetraacetic and tricarballylic acid.

It was found that the capacity of an acidic environment to decalcify enamel and dentin could be significantly increased or decreased by the addition of certain acid ions. In this respect enamel appears to be considerably more sensitive to the composition of the decalcification medium than dentin, whose solubility appears to be influenced only by those ions which show the strongest effects with enamel. Moreover, depending on the pH of the environment, the same ion can demonstrate opposite effects. As the pH increases from 2.6 to 6.0, the ions of citric acid and EDTA have no effect, then decrease, and finally increase the solubility rates of enamel in acid buffers. The increases in solubility observed with EDTA and citric acid are attributed to the ability of their ions at certain levels to form soluble calcium complexes, thus reducing the common ion effect which tends to suppress dissolution.

With respect to the solubility reductions observed, the following have been noted:

The monobasic acids produce little or no effect at any of the pH levels tested.

The dibasic acids demonstrate moderate effects.

The tribasic acids and EDTA show strong effects.

The presence of additional polar groups, such as amino or hydroxy, in these acids considerably enhance their capacity to reduce solubility.

From the contrasting findings with the homologues, aspartic and glutamic acids, and the sterioisomers, maleic and fumaric acids, there is an indication that the spatial arrangement of functional groups is important in determining the capacity for reduction.

The finding that the multicharged basic amino acid ions of lysine and ornithine, which have a net unit positive charge, produce reductions as strong as the ions of the acidic amino acid aspartic, which has a net unit negative charge, suggests that the nature of the charge is not so important as the existence of multiple charges on the ions.

It has been postulated that the ions which demonstrate reductions concentrate at or near the surfaces of the enamel and dentin crystallites, forming an ionic shell or surface-bound complexes that retard decalcification by interfering with the diffusion processes necessary for dissolution.

The possibility is suggested that specific ion effects of the sort presented may be of importance in biologic processes involving deposition and resorption of calcified tissues and the destruction of the teeth as occurs in caries.

REFERENCES

1. Armstrong, W. G., and Atkinson, H. F. Investigation into the Acidic Components of Carious Dentine, *Brit. D. J.*, **95**:8, 1953.
2. Kirch, E. R., *et al.* Amino Acids in Human Saliva, *J. D. Res.*, **26**:297, 1947.
3. Blackwell, R. Q., Fosdick, L. A., and Namajuska, I. Amino Acids in Dental Plaque, *J. D. Res.*, **33**:649, 1954 (abstr.).
4. Miller, B. G., and Muntz, J. A. The Quantity of Lactic Acid in Carious Areas of Human Teeth and Its Relationship to the Soluble Calcium and Phosphate in These Lesions, *J. D. Res.*, **18**:259, 1939 (abstr.).
5. Woldring, M. G. Free Amino Acids of Human Saliva: A Chromatographic Investigation, *J. D. Res.*, **34**:248, 1955.
6. Goldberg, H. J. V., Gilda, J. E., and Tishkoff, G. H. Paper Partition Chromatography: Free Amino Acids in Saliva, *J. D. Res.*, **27**:493, 1948.
7. Berry, H. K., and Cain, L. *Quantitative Study of Urinary and Salivary Amino Acids Using Paper Chromatology*, p. 71 (Univ. Texas Publ. No. 5109), 1951.
8. Shulman, E. H., and Robinson, H. B. G. Salivary Citrate Content and Erosion of the Teeth, *J. D. Res.*, **27**:541, 1948.
9. Muntz, J. A. Production of Acids from Glucose by Dental Plaque Material, *J. Biol. Chem.*, **148**:225, 1943.
10. Johnston, H. W. Chelation between Calcium and Organic Anions, *New Zealand J. Sci. and Tech.*, **37**:4, 1956.
11. ———. The Solubilisation of "Insoluble" Phosphate. II. A Quantitative and Comparative Study of the Action of Selected Aliphatic Acids or Tricalcium Phosphates, *ibid.*, **36**:49, 1954.
12. ———. The Solubilisation of "Insoluble" Phosphate. III. A Quantitative and Comparative Study of the Action of Chosen Aromatic Acids on Tricalcium Phosphate, *ibid.*, p. 281.
13. Osborn, T. W. B., Noriskin, J. N., and Staz, J. Inhibition *in Vitro* of Decalcification of Teeth, *J. D. Res.*, **16**:545, 1937.
14. Buonocore, M. G., and Sommer, B. The Effects of the Addition of Certain Amino Acids to Enamel Decalcifying Buffers, *J.A.D.A.*, **55**:781, 1957.
15. Jenkins, G. N. A Possible Mechanism of the Anti-Caries Action of Fluoride, *Odont. Revy*, **8**:68, 1957.
16. Manly, R., and Hodge, H. Density and Refractive Index Studies of Dental Hard Tissues, *J. D. Res.*, **18**:133, 1939.

17. Battistone, G. C., and Burnett, G. W. Studies of the Composition of Teeth. III. The Amino Acid Composition of Human Dentinal Protein, *J. D. Res.*, **35**:255, 1956.
18. Koulourides, T. A. The Effect of Organic Anions on Enamel and Dentin Solubility Rates in Acid Buffers. M. Sc. thesis, University of Rochester, 1958.
19. Buonocore, M. G. Dissolution Rates of Enamel and Dentin in Acid Buffers, *J. D. Res.*, **40**:561, 1961.
20. Apostolopoulos, X. A. Acid Dissolution Rates of Enamel, Dentin, and Bone. M. Sc. thesis, University of Rochester, 1960.
21. Albert, A. Quantitative Studies of the Acidity of Naturally Occurring Substances for Trace Metals, *Biochem. J.*, **50**:690, 1952.
22. Perkins, D. J. A Study of the Effect of Amino Acid Structure on the Stabilities of the Complexes Formed, *Biochem. J.*, **55**:649, 1953.
23. Taylor, T. G. The Nature of Bone Citrate, *Biochem. Biophys. Acta*, **39**:148, 1960.
24. Fosdick, L. S., Campaigne, E. E., and Fancher, O. Rate of Acid Formation in Carious Areas, *Ill. D. J.*, **10**:85, 1941.
25. Wach, E. C., Kesel, R. G., Hine, M. D., and O'Donnell, J. F. Testing Caries Activity by Acid Production in the Saliva, *J. D. Res.*, **22**:415, 1943.
26. Swerdlove, C. K. Relation between the Incidence of Dental Caries and the pH of Normal Resting Saliva, *J. D. Res.*, **21**:73, 1942.
27. Miller, B. F., Muntz, J. A., and Bradel, S. Decomposition of Carbohydrate Substrates by Dental Plaque Material, *J. D. Res.*, **19**:473, 1940.
28. Fosdick, L. W. A Correlation of Some Facts Concerning the Etiology and Control of Dental Caries, *J. Canad. D. A.*, **9**:259, 1943.
29. Bunting, R. W. Bacteriological Chemical and Nutritional Studies of Dental Caries by the Michigan Research Group, *J. D. Res.*, **14**:97, 1934.
30. Burrill, D. Y., and Fosdick, L. S. The Buffering Capacity and Rate of Flow of Saliva in Relation to Dental Caries, *N. W. Univ. Bull.*, **43**:17, 1942.

Dental Caries and Nutrition in Eskimo Scouts of the Alaska National Guard

A. L. RUSSELL, C. FRANK CONSOLAZIO, and CARL L. WHITE

National Institute of Dental Research, National Institutes of Health, United States Public Health Service, Bethesda, Maryland, and U.S. Army Medical Research and Nutrition Laboratory, Fitzsimmons Hospital, Denver, Colorado

Both high and low prevalence levels of dental caries have been reported for Alaskan natives. Leigh found little evidence of caries in 324 Eskimo skulls in the Smithsonian Institution.[1] Moorrees saw averages of 9 and 13 DMF teeth, respectively, in 46 male and 40 female Aleuts aged twelve through sixty-five years, from the villages of Nikolski and Atka on Umnak and Atka Islands.[2] Dental caries has been cited as an important public health problem in Alaskan Eskimos, partially on the experience of Point Barrow children; their DMF means were as high as, or higher than, those usually reported for children of continental United States at corresponding ages.[3] Unusually low caries prevalence was observed by Rosebury, Karshan, Siegel, and Waugh in 1936 and 1937, in studies carried out in the Yukon-Kuskokwin delta area.[4–6]

This report is a description of dental caries status and associated nutritive states of 713 members of the two Scout Battalions of the Alaska National Guard. The men were examined in March, 1958, in connection with a nutrition survey conducted by the Interdepartmental Committee on Nutrition for National Defense (the ICNND). This committee was constituted in 1955 to co-ordinate nutrition studies of friendly armed forces. Its field survey teams are made up of specialists in nutrition, medicine and dentistry, biochemistry, food technology, agriculture, and other disciplines as required by the specific situation.[7, 8] Preliminary reports of the findings have been presented elsewhere.[9, 10]

MATERIALS AND METHODS

In all, 713 men ranging in age from seventeen through fifty-four years were given oral examinations. They lived in fifty-five villages or towns throughout Alaska. Thirty-eight men came from two villages in the Aleutian chain of islands, and one group of 21 were Athabascan Indians from Fort Yukon. The remaining 654 men were about evenly divided between villages inhabited by northern and southern Eskimos. These are distinct ethnic groups with differing languages and cultures.[11]

Of the 8,200 Eskimo males in Alaska in 1950, about 3,700 were in the age range of seventeen through fifty-four years.[12] The sample examined, then, included about one Eskimo male in six for the entire state, or about one Eskimo male in five in the judicial areas represented. It included an even greater fraction of the able-bodied men. As Scott has pointed out, "a significant proportion of these [food-producing or wage-earning]

Received for publication December 16, 1960.

men had tuberculosis or other debilitating disease which made them unable to work or to hunt and fish."[13]

All the examinations were made in a mess hall at Camp Denali, Fort Richardson. The men were seated in a portable dental chair under a Castle examination lamp. Findings were dictated in code to an experienced recorder, following generally the procedures and criteria used by us in other dental caries studies.[14] Each tooth was recorded as free of caries, or decayed, or missing or filled. Frank loss of tooth substance was required before a lesion was designated as carious. A tooth with a crown so destroyed by caries that filling was deemed impracticable was recorded as indicated for extraction and tabulated as a missing tooth. A tooth with a filling and a separate carious lesion was counted, once only, as a filled tooth. In many cases arches were so crowded that no room remained for eruption of one or more bicuspids. These teeth were considered to be unerupted rather than missing if the crown could be palpated below the gingivae at the margin of the alveolar crest.

Each permanent molar tooth was examined for signs of attrition and scored on the accompanying scale. The total of these scores was divided by the number of molar teeth present and the result entered as the attrition score for that individual.

Observation	Tooth Score
No signs of attrition	0
Hard, shiny facets on enamel	1
Dentin exposed on occlusal surface	2
Raised cusps eliminated; occlusal surface essentially horizontal	3
Maximum height of crown of tooth above amelocemental junction on buccal aspect less than 3 mm.	4

These men were also examined for evidences of past and present periodontal disease, the presence or absence of material alba or calculus, and notes were entered describing arch form, occlusion, anomalies of enamel, and the like. These findings will be presented in separate reports.

On the day of examination, fasting blood and fasting 6-hour urine samples were collected. Blood samples were taken in two vacutainer tubes, a 20-ml. size for whole clotted blood and a 10-ml. oxalated tube containing 20 mg. of the oxalate mixture per tube for hemoglobin analysis. The oxalated blood specimens were analyzed daily for hemoglobin, plasma protein, and ascorbic acid at the Arctic Health Research Center in Anchorage. Sera from the whole blood samples were frozen for analysis elsewhere.

The 6-hour urine volumes were measured, with a 60-ml. aliquot being acidified with 0.1 ml. of concentrated hydrochloric acid and immediately frozen. The frozen serum and urine specimens were packed in dry ice and shipped by air express to the U.S. Army Medical Research and Nutrition Laboratory in Denver, where they usually arrived within 20 hours, still in the frozen state. There the serum was analyzed for vitamin A and carotene, and the urine was analyzed for thiamine, riboflavin, *N'*methylnicotinamide, and creatinine. The methods were those listed as standard in the ICNND *Manual*.[15]

RESULTS

The men seemed generally to be well nourished and in excellent physical condition.[9] Numbers of decayed, missing, and filled permanent teeth in the whole group examined

are shown in Table 1. For comparison similar data are shown for a quasi-random sample of males examined in Baltimore in 1954.[16] Identical criteria were followed in these studies, and the Alaska dental examiner participated in both. Caries prevalence in Alaskan Scouts was importantly lower than prevalence in white males in Baltimore, though about the same as that in Negro males at the younger ages. Both sets of Baltimore data show the usual increase of DMF means with age. In Alaska Scouts, on the contrary, DMF findings dwindle from about 10 per man in the group aged seventeen

TABLE 1

DENTAL CARIES IN MEN OF THE SCOUT BATTALIONS, ALASKA NATIONAL GUARD, 1958

Age Group	No. Examined	Mean Age	Mean Numbers of Permanent Teeth				Mean DMF, Baltimore	
			Decayed	Missing	Filled	DMF	White Males	Negro Males
17–19	63	18.9	2.6	4.7	2.9	10.2±0.85	12.9	8.9
20–24	192	22.3	2.2	5.3	1.5	9.1±0.53	13.0	7.2
25–29	167	27.4	1.8	6.7	1.4	9.8±0.66	14.4	7.5
30–34	128	32.4	2.0	5.5	1.0	8.5±0.67	14.2	8.0
35–39	86	37.4	1.4	4.6	0.6	6.6±0.81	15.5	9.9
40–44	45	42.5	0.7	5.6	0.3	6.6±1.27	18.0	11.2
45 and over	32	48.4	0.4	5.5	0.8	6.8±2.52	18.6	15.2
Total	713	29.3	1.9	5.5	1.3	8.7±0.29	.....	.....

TABLE 2

MEAN NUMBERS OF DMF TEETH IN NORTHERN AND SOUTHERN ESKIMOS OF THE ALASKA SCOUT BATTALIONS, 1958

Age Group (Years)	Northern Eskimos		Southern Eskimos		P
	No. Examined	Mean DMF	No. Examined	Mean DMF	
17–24	145	10.8	88	6.4	<0.001
25–34	165	10.7	106	5.1	<0.001
35 and over	59	9.2	91	3.5	<0.001
All ages	369	10.5	285	5.0	<0.001

to nineteen years to between 6 and 7 per man at all ages over thirty-five years. Since the number of decayed, missing, and filled permanent teeth per man is an irreversible indication of lifetime caries experience, this would indicate that caries is much more active in the present group aged seventeen to nineteen years than it was in oldest men of the sample at the time they were seventeen to nineteen years of age.

Little difference was seen in the nutritive status of men in the First as compared with men of the Second Scout Battalion. These were composed principally of men from the northern and southern ethnic groups, respectively. Their nutritional status has been reported in detail elsewhere.[9] As shown in Table 2, dental caries prevalence was significantly greater in northern Eskimo men. About 7 per cent of northern men were caries-

free, compared with 29 per cent of men from the southern areas. While this might suggest that caries prevalence was related to ethnic origin, it was obvious from the outset that prevalence was related much more closely to place of residence; a preliminary allocation of villages to high-, medium-, and low-caries groups (before the location of any of the places was known) was found to have combined them into clusters with definable geographic boundaries. The highest means were found for men in the seven principal villages and for men of eleven other places relatively close to these principal centers. Intermediate DMF means were found in men from villages which, considered as a group, tended to be relatively remote from these principal centers. The low-caries villages, with a single exception, were found in the tundra region of the Kuskokwim-Yukon delta area and on nearby Nunivak Island. There seemed to be logical subdivisions for comparisons with group nutritive states.*

Mean numbers of DMF teeth for these groups are shown in Table 3. Group I means rise slightly with age; group II means decline slightly; group III means not only are the lowest of the array but show the most marked tendency to decline with increasing age. Essentially the same patterns are seen in the proportions of men who were caries-free. The findings for group I do not differ importantly from those for white males in Baltimore. Those for groups II and III differ markedly, both in magnitude and in the slope of association with age.

Not all biochemical and physical examination details were recorded for each man in the sample. For 503 men, data on height, weight, plasma protein, hemoglobin, serum vitamin C, serum vitamin A, serum carotene, urinary thiamine, urinary riboflavin, urinary *N'*methylnicotinamide, and urinary creatinine were complete. DMF data and distribution to groups were essentially the same in this smaller sample as in the whole number of men who received dental examination. These findings for the groups, compared with their lifetime caries experience, are shown in Table 4. Approximate probabilities are indicated, based on *F*-ratios. Within-groups degrees of freedom were 186, 209, 99, and 500, respectively.

Men with the higher caries experience were taller and heavier, particularly at ages up to twenty-four years, and showed significantly higher levels of plasma vitamin A and carotene. Men with low caries experience were excreting substantially higher amounts of urinary *N'*methylnicotinamide, though most of the differences was found in men aged thirty-five years or over. Differences between groups were generally of little significance in other factors, with great variation from individual to individual, regardless of caries experience.

* Composition of the groups by village was as follows:

Group I—principal villages, 164 men: Bethel, Barrow, Kotzebue, Dillingham, Unalaska, Nome, and St. Paul. Nearby villages, 100 men: Noatak, Deering, Tuluksak, Wainwright, Napaskiak, Akiak, White Mountain, Shishmaref, Unalakleet, Elim, Shaktoolik.

Group II—relatively remote villages (except the Kuskokwim-Yukon area), 297 men: Little Diomede, Shungnak, Kasigluk, Akiachak, Barker Island, Alakanuk, Teller plus Teller Mission, Koyuk, Kwethluk, Stebbins, Selawik, Meade River, Eek, Mountain Village, Wales, Kivalina, Gambell, Kiana, Noorvik, Fort Yukon, Savoonga, King Island, St. Michael, and Point Hope.

Group III—villages in the Yukon-Kuskokwim delta area, 152 men: Newtok, Chevak, Tununak, Mekoryuk, Chefornak, Kwillingnak, Kipnuk, Hooper Bay, Tuntutuliak, Quinhagak, Napaskiak, Togiak, and Scammon Bay.

Individual village DMF means, periodontal scores and recession scores were published in the original team report of the ICNND (see ref. 9).

Mean molar attrition scores are shown in Table 5. At the youngest ages, scores in group I were somewhat lower, and scores in groups II and III somewhat higher, than attrition scores reported for Chinese, Malaysians, Indians, and Pakistani of the same ages in Singapore by McCombie and Chua.[17] There was a general, though weak, association between caries experience and attrition, with the lower caries experience tending to accompany the higher degree of attrition. But this association was too erratic for prediction of the one value from the other, even for group means; mean attrition scores were virtually the same for men of groups II and III aged seventeen to twenty-four years, and for men of groups I and II aged twenty-five to thirty-four years, despite wide differences in caries experience between these groups.

TABLE 3

DENTAL CARIES STATUS OF ALASKAN ESKIMO SCOUTS IN THREE GROUPS OF VILLAGES

Age Groups (Years)	Group I	Group II	Group III
	Men Living in 7 Principal Villages or in 11 Nearby Villages	Men Living in 24 Relatively Remote Villages, Except Following Group	Men Living in 13 Villages in Yukon-Kuskokwim Delta Area
	Numbers Examined		
17–24	110	104	41
25–34	116	125	54
35 and over	38	68	57
All ages	264	297	152
	Mean Numbers of DMF Teeth		
17–24	13.2±0.68	7.6±0.57	3.5±0.64
25–34	14.8±0.75	7.0±0.56	2.4±0.44
35 and over	15.0±1.46	6.5±0.70	1.3±0.32
All ages	14.2±0.48	7.1±0.35	2.3±0.27
	Per Cent of Men Caries-free		
17–24	3±1.6	7±2.5	34±7.4
25–34	3±1.6	13±3.0	48±6.8
35 and over	3±2.8	12±3.9	56±6.6
All ages	3±1.0	10±1.7	47±4.0
	Per Cent of Men Edentulous		
17–24	2±1.3	0	0
25–34	9±2.7	2±1.3	0
35 and over	16±5.9	0	0
All ages	7±1.6	1±0.6	0

DISCUSSION

Scott has said:

> Today there are probably no Eskimos in Alaska who subsist on wholly native diets. The most that can be said is that certain people at certain times live wholly from native foods. . . . The choice of food available in the village stores is limited because the stores receive their principal shipments by boat only twice a year, and many other foods must be shipped in by air at considerable cost. The stores in general lack suitable storage facilities, both where foods can be kept frozen and where freezing can be avoided.[13]

At the time of these examinations, in March, stores of imported foods were nearly depleted, and men in the remote villages were subsisting on essentially the traditional native diet. There is little evidence in these findings to indicate that their nutritive status had deteriorated as a result. Neither is there any evidence that their relative freedom from dental caries is due to any superiority in any of the nutritional elements considered in this study.

No fluorosis was seen in any of the men. Fluoride ingestion, hence, seems inadmissible as the reason for the low caries prevalence levels.

An association between primitive conditions of living, or isolation, and dental caries prevalence has been reported for such diverse peoples as Greenland Eskimos,[18] Polynesians of Pukapuka and Rarotonga,[19] Bantu of South Africa,[20] and Greek children of Kitta.[21] In the present instance, some of the "nearby" villages included in group I are actually more distant from a principal town than a few of the "relatively remote" villages in group II; there is a great deal of overlap in these two categories. Some assignments of borderline villages were arbitrary, and some assignments were probably influenced by sampling error. Nonetheless, no manipulation of doubtful assignments makes any essential change in the broad pattern of disease—a progressive diminution of caries prevalence with progressive isolation from the "principal" centers of population.

Neither can the term "isolation" be accepted in any absolute sense. Men from all the villages except Shungnak usually leave their homes during the summer for paid employment, hunting, or fishing. Whole villages in the Norton Sound area, for example, normally migrate to Nome during the warm season. These villages range from Shishmaref on the north to St. Michael and Stebbins on the south. Mean DMF for men of Nome was 14.5 ± 1.8. DMF means for men of the 10 villages which annually gather in Nome varied from 5.6 ± 1.5 to 21.5 ± 1.5. The odds are about one in 1,000 that these are not samples from the same universe. The caries-inhibitory factors associated with "primitiveness" or "isolation" hence seem to be independent here of a yearly shoulder-to-shoulder contact with men from the more acculturated villages, and access, during this period at least to essentially the same types of food.

If the Rosebury and Waugh data of 1936[4] are taken as a base line, the increase in caries prevalence in the Yukon-Kuskokwim area over the past two decades has been somewhat less than might be inferred from the data for group III men shown in Table 3. Prevalences reported by Rosebury and Waugh are compared with prevalences elicited from the 1958 data in Table 6. The 1936 data include findings for females, the population groups averaged about 10 years younger, and the examiners were not aided by artificial light. In view of these differences and possible differences in criteria, the apparent rise in absolute prevalences may be spurious. But even if these differences are

TABLE 4

DENTAL CARIES EXPERIENCE AND NUTRITIVE STATUS IN THREE GROUPS OF ALASKAN ESKIMO SCOUTS

AGES	CARIES GROUPS			F	P
	I	II	III		
	Mean Numbers of DMF Teeth per Man				
17–24	13.6	8.3	2.7	33.175	<0.001
25–34	14.7	7.2	2.6	46.160	<0.001
35–54	14.4	6.9	1.4	30.559	<0.001
All ages	14.2	7.5	2.1	118.068	<0.001
	Mean Height (in Inches)				
17–24	66.0	65.6	64.1	8.825	<0.001
25–34	66.2	65.7	64.5	4.827	<0.01
35–54	64.9	65.2	64.4	0.582	>0.20
All ages	66.0	65.5	64.4	15.667	<0.001
	Mean Weight (in Pounds)				
17–24	144.5	145.3	135.8	4.512	0.01
25–34	151.4	146.6	143.9	3.118	0.05
35–54	149.9	145.3	146.9	0.677	>0.20
All ages	148.3	145.9	142.6	4.272	0.02
	Total Plasma Protein (in Gm/100 Ml)				
17–24	7.2	7.1	7.1	3.503	0.04
25–34	7.2	7.1	7.2	0.915	>0.20
35–54	7.1	7.1	7.1	0.110	>0.20
All ages	7.2	7.1	7.1	2.107	0.13
	Hemoglobin (in Gm/100 Ml)				
17–24	14.7	14.7	14.7	0.007	>0.20
25–34	14.6	14.7	14.4	1.651	0.20
35–54	14.8	14.4	14.2	3.663	0.03
All ages	14.6	14.6	14.4	2.613	0.08
	Serum Ascorbic Acid (in Mg/100 Ml)				
17–24	0.55	0.50	0.50	0.727	>0.20
25–34	0.51	0.52	0.57	0.315	>0.20
35–54	0.53	0.54	0.59	0.357	>0.20
All ages	0.53	0.52	0.56	0.521	>0.20

TABLE 4—*Continued*

Ages	Caries Groups			*F*	*P*
	I	II	III		
	Serum Vitamin A (in μg/100 Ml)				
17–24	42	37	36	4.416	0.01
25–34	39	34	33	4.764	0.01
35–54	41	36	35	0.896	>0.20
All ages	40	36	35	9.616	<0.001
	Serum Carotene (in μg/100 Ml)				
17–24	125	100	97	13.905	<0.001
25–34	121	95	100	13.763	<0.001
35–54	135	94	101	9.971	<0.001
All ages	124	96	99	36.741	<0.001
	Urinary Thiamine (in μg/Gm Creatinine)				
17–24	270	287	297	0.195	>0.20
25–34	305	255	231	1.955	0.15
35–54	261	211	203	1.024	>0.20
All ages	286	258	241	1.835	0.17
	Urinary Riboflavin (in μg/Gm Creatinine)				
17–24	751	683	730	0.295	>0.20
25–34	711	676	642	0.153	>0.20
35–54	825	714	612	1.555	>0.20
All ages	740	686	657	1.070	>0.20
	Urinary *N'*Methylnicotinamide (in Mg/Gm Creatinine)				
17–24	12.8	14.9	18.2	1.043	>0.20
25–34	12.8	15.7	14.8	1.518	>0.20
35–54	8.6	14.9	14.3	3.307	0.04
All ages	12.3	15.3	15.7	2.922	0.05

TABLE 5

MEAN MOLAR ATTRITION SCORES IN ALASKAN SCOUTS WITH HIGH, MODERATE, AND LOW LIFETIME CARIES EXPERIENCE

Ages	Group I (High Caries)	Group II (Moderate Caries)	Group III (Low Caries)
17–24	0.45±0.06	0.65±0.04	0.64±0.09
25–34	1.00±0.07	1.05±0.05	1.41±0.09
35–54	1.53±0.16	1.85±0.08	2.24±0.10

accepted at face value, it is evident that any increase in caries prevalence over this time span has been gradual rather than explosive and that prevalence in the highest of these groups—men living in and near Bethel—would be considered to be unusually low if observed in males of the same ages living in the central United States.

In the present case the more isolated groups had, in general, tended to exhaust their stores of imported foods earlier and to have been subsisting for a longer time more nearly on their traditional diets than men from the principal centers of population. Biochemical and nutritive findings for men of the more isolated places, then, may reflect the nutritive states associated with these traditional diets with considerable accuracy.

TABLE 6

DMF TEETH IN ALASKAN ESKIMOS OF KUSKOKWIM AREA, 1936 AND 1938

REGION	No. EXAMINED	MEAN AGE	MEAN NUMBERS OF PERMANENT TEETH PER PERSON				
			Decayed	Missing	Extraction Indicated	Filled	DMF
	Rosebury and Waugh, 1936; Male and Female						
Bethel and vicinity*	47	16.3	5.03	1.28	.....	0.26	6.57
Eek	20	18.4	4.35	0.15	.....	0	4.50
Primitive villages†	39	21.7	0.49	0.13	.....	0	0.62
Kepnuk	9	21.6	0.33	0	.....	0	0.33
	1958; Male Only						
Bethel and vicinity*	82	29.4	2.43	4.57	0.78	0.70	8.48±0.79
Eek	14	28.7	1.29	3.29	0.43	1.86	6.86±1.78
Primitive villages†	152	31.7	0.76	1.13	0.30	0.12	2.30±0.27
Kipnuk	13	30.8	0.38	1.08	0.15	0.38	2.00±0.60

* In 1936 "this includes Kwithluk, Akiak, Kalsag, and the tundra villages west of Bethel." In 1958 the villages included are Bethel, Kwethluk, Akiak, Kasigluk, Napaskiak, Tuluksak, and Akiachak.

† In 1936 "this includes Napakiak, Quigillingnok, Kepnuk, Nelson Island, Nunivak Island and other coastal villages." In 1958 the villages included are Chefornak, Chevak, Hooper Bay, Kipnuk, Kwillingnak, Mekoryuk (Nunivak Island), Napakiak, Newktok, Quinhagok, Scammon Bay, Togiak, Tuntutuliak, and Tununak.

These were the men with by far the lowest lifetime experience of dental caries. In most respects these men were slightly lower in indicators of nutritional status than men with higher caries experience. The findings support the conclusion of Siegel, Waugh, and Karshan that "there is no basis for the view frequently held that the freedom from caries enjoyed by a primitive people is due to a nutritional superiority of 'natural' as compared with 'civilized' diets."[6]

SUMMARY

Dental caries examinations were given 713 men of the two Scout Battalions of the Alaska National Guard in March, 1958. Biochemical determination of the levels of certain nutrients were made for a large fraction of this sample.

Dental caries prevalence was found to be high in the principal villages and some of the villages nearby; intermediate in prevalence in men from relatively remote villages;

and very low in men from coastal villages in the Kuskokwim-Yukon delta area. In the last two groups prevalence seemed to be rising.

Men in the high-caries villages tended to be somewhat taller and heavier, to show somewhat higher levels of serum carotene and serum vitamin A, and somewhat lower levels of urinary *N'*methynicotinamide. There were no differences associated with caries in total plasma protein, hemoglobin, and ascorbic acid, or in urinary thiamine or riboflavin.

These findings would tend to support the earlier conclusion of Siegel, Waugh, and Karshan that relative freedom from caries in the more primitive groups is not due to any nutritional superiority of the traditional diet.

REFERENCES

1. Leigh, R. W. Dental Pathology in the Eskimo, *D. Cosmos,* September, 1925.
2. Moorrees, C. F. A. *The Aleut Dentition: A Correlative Study of Dental Characteristics in an Eskimoid People.* Cambridge: Harvard University Press, 1957.
3. Parran, Thomas, *et al. Alaska's Health: A Survey Report to the U.S. Department of the Interior by the Alaska Health Survey Team.* Pittsburgh: University of Pittsburgh, 1954.
4. Rosebury, Theodor, and Waugh, L. M. Dental Caries among Eskimos of the Kuskokwim Area of Alaska. I. Clinical and Bacteriological Findings, *Am. J. Diseases Children,* **57**:871, 1939.
5. Rosebury, Theodor, and Karshan, Maxwell. Dental Caries among Eskimos of the Kuskokwim Area of Alaska. III. A Dietary Study of Three Eskimo Settlements, *Am. J. Diseases Children,* **57**:1343, 1939.
6. Siegel, E. H., Waugh, L. M., and Karshan, Maxwell. Dietary and Metabolic Studies of Eskimo Children with and without Dental Caries, *Am. J. Diseases Children,* **59**:19, 1940.
7. Berry, F. B., and Schaefer, A. E. Program of the Interdepartmental Committee on Nutrition for National Defense (Report to the Council on Foods and Nutrition), *J. Am. Med. Assoc.,* **166**: 775, 1958.
8. Schaefer, A. E. Interdepartmental Committee on Nutrition for National Defense, *Nutrition Rev.,* **16**:193, 1958.
9. Interdepartmental Committee on Nutrition for National Defense. *Alaska: An Appraisal of the Health and Nutritional Status of the Eskimo.* Bethesda, 1959.
10. Russell, A. L., Littleton, N .W., Leatherwood, E. D., Sydow, G. E., and Greene, J. C. Dental Surveys in Relation to Nutrition, *Pub Health Repts.,* **75**:717, 1960.
11. Lantis, Margaret. *Alaskan Eskimo Ceremonialism.* New York: J. J. Augustin (American Ethnological Society), 1947.
12. U.S. Department of Commerce, Bureau of the Census. *Census of Population: 1950,* Vol. II: *Characteristics of the Population,* Parts 51–54; *Territories and Possessions,* Table 29, pp. 51–36. Washington: Government Printing Office, 1953.
13. Scott, E. M. Nutrition of Alaskan Eskimos, *Nutrition Rev.,* **14**:1, 1956.
14. Russell, A. L. Oral Health Study in Children of Suburban Washington, D.C., *Pub. Health Repts.,* **71**:626, 1956.
15. Interdepartmental Committee on Nutrition for National Defense. *Manual for Nutrition Surveys.* Washington: Government Printing Office, 1957.
16. Prevalence of Dental Conditions. In Commission on Chronic Illness, *Chronic Illness in a Large City,* pp. 107–18. Cambridge: Harvard University Press, 1957.
17. McCombie, F., and Chua, S. C. Dental Epidemiology in Malaya. II. A Survey of Chinese, Malay, and Indian Young Adult Males in Singapore, *Canad. D. A. J.,* **23**:687, 1957.
18. Baarregaard, Anker. Dental Conditions and Nutrition among Natives of Greenland, *Oral Surg., Oral Med. Oral Path.,* **2**:995, 1949.
19. Davies, G. N. A Comparative Epidemiological Study of the Diet and Dental Caries in Three Isolated Communities. In J. F. Volker, Chairman, *Conference on Diet and Oral Health,* pp. 19–44. Birmingham: University of Alabama, 1956.
20. Oranje, P., Noriskin, J. N., and Osborn, T. W. B. The Effect of Diet upon Dental Caries in the African Bantu, *S. African J. Med. Sci.,* **1**:57, 1935.
21. Philippas, G. C. Dental Caries among a Group of Greek Children, *J.A.D.A.,* **50**:539, 1955.

Periodontal Disease and Nutrition in Eskimo Scouts of the Alaska National Guard

A. L. RUSSELL, C. FRANK CONSOLAZIO, and CARL L. WHITE

National Institute of Dental Research, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland, and U.S. Army Medical Research and Nutrition Laboratory, Fitzsimmons Hospital, Denver, Colorado

Data from an earlier study seemed to indicate that Alabama people with the better educational attainment showed a better condition of the periodontal tissues than could readily be explained by their more effective oral hygiene and their more adequate professional care.[1] One possible factor, not explored in the Alabama study, was nutrition. This is a report of nutritive states and associated periodontal findings in members of the first and second Scout Battalions of the Alaska National Guard, groups in which the influence of oral hygiene and professional dental care was negligible. Dental caries findings for these groups have been described in an earlier paper.[2]

MATERIALS AND METHODS

The men were examined in March, 1958, at Fort Richardson, near Anchorage. Four distinct ethnic groups were represented. Two groups were small, including 21 Athabascan Indians from Fort Yukon and 38 Aleuts from Unalaska and St. Paul. Of the remainder, 369 men were northern and 285 were southern Eskimos. These are distinct ethnic groups with differing languages and culture.[3] There were some differences in the traditional diet of these Eskimo groups, related more to the availability of food than to culture. In the areas occupied generally by northern Eskimos,

> . . . the major proportion of food was obtained from seal, walrus, and whale. These were supplemented by birds, eggs, fish, shellfish, and plants in small amounts. Dependence upon these marine mammals had its risks, since they tend to be migratory, and famines occurred in this area.
>
> Further south, the principal source of calories was fish, which were abundant in the rivers and tundra ponds. This was supplemented by small mammals, including mink, muskrat and rabbit, birds, eggs and plants. The large fish including salmon, pike, several varieties of whitefish, and ling cod were all eaten in large amounts, while four smaller varieties, the blackfish, eulachon, smelt and stickleback could be easily caught in large quantity. Where fish were abundant, they appeared to be a more reliable food supply than were sea mammals.
>
> . . . these diets and their variants had common characteristics, in that they were low in plant foods, carbohydrate, calcium, and vitamin C. The diets were all high in protein, but not necessarily high in fat.[4]

The study was part of a nutrition survey conducted by the Interdepartmental Committee on Nutrition for National Defense.[5] The men were given a general physical and a dental examination, and biochemical determinations were made of hemoglobin, total

Received for publication December 16, 1960.

plasma protein, serum ascorbic acid, serum vitamin A and carotene, and of thiamine, ribofiavin, and *N'*methinicotinamide in urine.

The dental examination was conducted with mirror and explorer, with the man seated in a portable dental chair under a standard examination lamp. In addition to findings for dental caries and molar attrition previously described,[2] the men were examined for the presence of materia alba on the gingival third of the labial surface of two or more incisor teeth (as demonstrated by scraping with explorer tine) and the presence of supragingival calculus on the crowns of two or more teeth anywhere in the mouth. Notes were entered describing arch form, occlusion, and anomalies of the enamel. Present and active peridontal disease was scored by means of the Periodontal Index (PI),[6] and cumulative evidence of bone resorption as the mean per cent per man of teeth showing gingival recession past the cemento-enamel junction, as used by Stahl and Morris.[7]

TABLE 1

PERIODONTAL AND GINGIVAL RECESSION SCORES IN 693 MEN OF THE ALASKA SCOUT BATTALIONS, 1958

AGE GROUP (YEARS)	NO. EXAMINED	MEAN AGE	PERIODONTAL SCORE	RECESSION SCORE	PERIODONTAL SCORES, BALTIMORE		RECESSION SCORES, U.S. ARMY ENGINEERS*
					White Male	Negro Male	
17–19.....	63	18.8	0.40±0.06	1.6±0.7	0.27	0.42	7.2
20–24.....	190	22.3	0.53±0.05	5.2±0.9	0.24	0.47	9.4
25–29.....	158	27.5	0.88±0.09	10.9±1.3	0.30	0.28	13.1
30–34.....	125	32.4	1.23±0.13	16.9±1.7	0.81	1.09	17.1
35–39.....	84	37.4	1.64±0.17	24.5±2.6	0.84	1.25	21.8
40–44.....	42	42.5	1.36±0.23	27.7±4.4	1.11	2.33	27.1
45 and over..	31	48.2	1.47±0.35	26.1±4.5	1.32	2.18	33.7

* Computed for these age points from findings of Stahl and Morris (see ref. 7).

RESULTS

Twenty of the men were edentulous, leaving 693 who could be scored for periodontal disease and gingival recession. These scores are shown in Table 1, together with periodontal scores for males of the same ages examined in Baltimore in 1954[8] and recession scores computed for the corresponding mean ages from data on U.S. Army Engineers reported by Stahl and Morris.[7] PI scores were higher in all age groups than those for white males in Baltimore, though differences in men forty years of age or older are of little clinical significance. At these ages, PI scores for Negro males in Baltimore were importantly higher than in the Eskimo men. Recession scores were slightly below those based on findings for Army Engineers. Group PI and group recession scores were so highly correlated that the one could be estimated from the other with negligible error.

Dental caries prevalence had been found to be somewhat higher in northern than in southern Eskimos and sharply different, depending on whether the men lived in or near one of the principal villages, in a somewhat more remote village, or on the tundra of the Yukon-Kuskokwim area.[2] When periodontal disease data were contrasted for these same geographical groups, no differences of clinical or statistical significance appeared

TABLE 2

NUTRITIONAL AND PERIODONTAL STATUS OF THREE GROUPS OF ALASKAN ESKIMO SCOUTS

AGE GROUP (YEARS)	GROUP A	GROUP B	GROUP C	APPROX. *P**
	Mean Periodontal Scores			
17–24	0.27	0.50	0.59	0.08
25–34	0.41	1.10	1.23	0.01
35 and over	0.49	1.43	2.27	<0.001
All ages	0.38	0.95	1.09	<0.001
	Mean Per Cent Recession			
17–24	0.9	2.1	7.9	<0.001
25–34	8.7	11.2	17.6	0.02
35 and over	14.4	18.1	46.7	<0.001
All ages	7.3	9.5	16.8	<0.001
	Mean Height (in Inches)			
17–24	65.9	65.2	65.8	0.16
25–34	67.3	64.9	65.7	<0.001
35 and over	65.5	64.1	65.8	<0.01
All ages	66.3	64.8	65.8	<0.001
	Mean Weight (in Pounds)			
17–24	142.3	140.1	147.8	<0.01
25–34	153.4	144.7	148.7	0.05
35 and over	150.5	143.8	149.0	0.10
All ages	148.5	142.6	148.4	<0.001
	Total Plasma Protein (in Gm/100 Ml)			
17–24	7.1	7.2	7.1	>0.20
25–34	7.1	7.1	7.2	>0.20
35 and over	7.1	7.1	7.2	0.11
All ages	7.1	7.1	7.2	>0.20
	Hemoglobin (in Gm/100 Ml)			
17–24	14.6	14.8	14.7	>0.20
25–34	14.8	14.4	14.6	0.09
35 and over	14.3	14.2	14.7	0.10
All ages	14.6	14.5	14.6	>0.20

* Based on *F*-ratios.

TABLE 2—*Continued*

Age Group (Years)	Group A	Group B	Group C	Approx. *P**
	Serum Ascorbic Acid (in Mg/100 Ml)			
17–24	0.48	0.53	0.52	>0.20
25–34	0.59	0.49	0.53	>0.20
35 and over	0.54	0.56	0.56	>0.20
All ages	0.54	0.53	0.53	>0.20
	Serum Vitamin A (in μg/100 Ml)			
17–24	35.1	40.3	38.3	0.15
25–34	31.6	37.8	36.1	0.09
35 and over	34.6	35.7	41.2	>0.20
All ages	33.7	38.2	37.4	0.04
	Serum Carotene (in μg/100 Ml)			
17–24	100.5	119.6	103.3	0.007
25–34	107.9	104.8	104.8	>0.20
35 and over	95.8	105.9	98.7	>0.20
All ages	101.9	110.9	103.6	0.07
	Urinary Thiamine (in μg/Gm Creatinine)			
17–24	298.0	298.0	262.8	>0.20
25–34	327.9	336.3	214.8	<0.001
35 and over	167.9	237.1	191.9	0.14
All ages	274.3	294.5	230.9	0.01
	Urinary Riboflavin (in μg/Gm Creatinine)			
17–24	718.0	776.9	667.7	>0.20
25–34	732.9	718.0	654.4	>0.20
35 and over	583.3	684.3	863.0	0.12
All ages	687.7	732.0	680.6	>0.20
	Urinary *N'*Methylnicotinamide (in Mg/Gm Creatinine)			
17–24	13.9	16.7	12.7	0.20
25–34	15.5	16.5	12.9	0.12
35 and over	11.8	16.0	9.9	0.04
All ages	13.9	16.5	12.5	0.02

in men up through the age of thirty-four years. In older men, both PI and mean recession scores were significantly higher in northern than in southern Eskimos and were closely related to mean DMF findings; DMF means were 15.0, 6.5, and 1.3 for men of the three geographic groups; mean PI scores were 1.99, 1.72, and 1.05; and mean recession scores were 34.9, 28.2, and 17.5, respectively. The nutritional status of these men as reported in the earlier publication[2] is essentially applicable here, despite some small difference in numbers due to the exclusion of edentulous men. Hemoglobin and serum carotene levels were slightly, but significantly, higher in the group with the highest prevalence of periodontal disease; the reverse was true of urinary excretory levels of *N'*methylnicotinamide. Differences in height, weight, total plasma protein, serum ascorbic acid and vitamin A, and urinary thiamine and riboflavin were small, inconsistent, and not significant statistically. But the numbers of men examined were small in this age group, and an association with nutritive states seemed more likely to be discovered, if one existed, if larger groups with wider differences in disease status could be brought to contrast.

Accordingly, mean PI and recession scores and mean ages were computed for whole-village groups and compared with PI scores for males in Baltimore (white and Negro combined) and with recession scores as computed for U.S. Army Engineers. Three categories were set up on the basis of these comparisons. Men from villages with whole-group scores lower in both PI and recession means than United States base lines were placed in group A; men from villages higher in both respects in Group C; and men from villages scoring higher by one index and lower by the other were placed in group B.*

Of the 26 villages in group B, mean PI scores were higher than those in Baltimore, and recession scores lower in 25. This seemed consistent with the clinical impression at the time of examination that inordinate numbers of men with inflammation of the gingivae showed little evidence of bone resorption.

Periodontal and recession scores for these groups are shown in Table 2 and in Figures 1 and 2. Table 2 likewise shows the nutritive status of the men. Data are limited to the 485 men for whom biochemical determinations were complete. Within-group degrees of freedom were 183, 197, 93, and 481, respectively.

Group A is composed of men relatively free of signs of past or present disease. They tended to be slightly, but significantly, taller and heavier than men of the other two groups and showed generally lower levels of serum vitamin A. Group C, men with signs of relatively extensive and severe past and present disease, seems to differ only in a tendency toward lower levels of urinary thiamine. Men of group B, in whom gingival disease presumably of long standing is associated with no more than minimal

* Group A villages (83 men): Mekoryuk, Scammon Bay, Fort Yukon, Tununak, Kiana, Shungnak, Chefornak, Dillingham, Barter Island, Selawik, Teller, and Shishmaref.

Group B villages (204 men): Noorvik, Meade River, Bethel, Nome, Noatak, Elim, Kipnuk, Napakiak, Kasigluk, Unalaska, Newktok, Hooper Bay, White Mountain, Mountain Village, Quinhagak, Point Hope, Wales, Eek, Kwillingnak, Akiak, Tuntutuliak, St. Paul, King Island, Kwethluk, Togiak, and Alakanuk.

Group C villages (198 men): Savoonga, Barrow, Gambell, Unalakleet, Kotzebue, Shaktoolik, Napaskiak, Little Diomede, Chevak, Kivalina, Deering, Wainwright, Tuluksak, Stabbins, St. Michael, Koyuk, and Akiachak.

There is no association between these and the groups designated I, II, and III used for analysis of dental caries status.

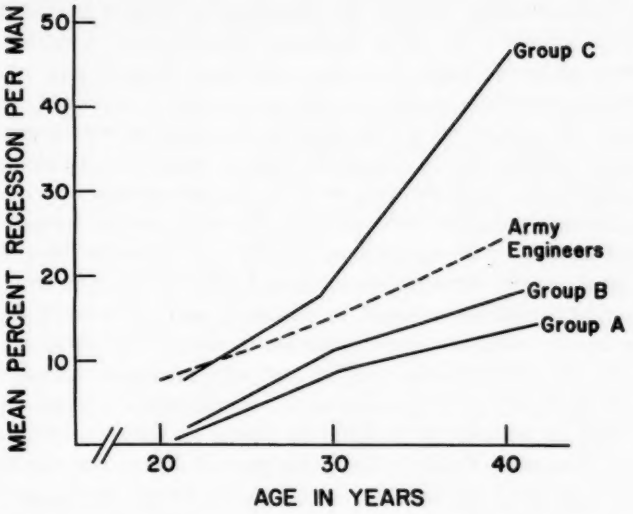


FIG. 1.—Mean gingival recession scores in three groups of Alaskan Scouts and Army Engineers

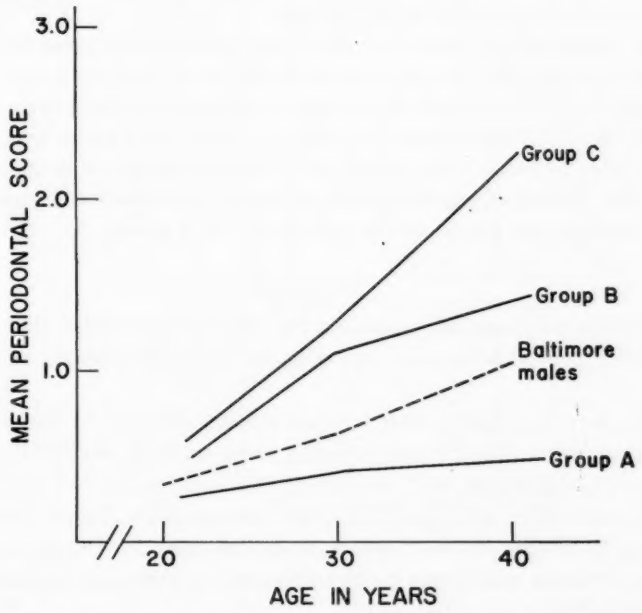


FIG. 2.—Mean periodontal scores in three groups of Alaskan Scouts and Baltimore males

signs of bone resorption, are slightly, but significantly, smaller and lighter in weight than men of groups A and C and show somewhat higher levels of *N'*methylnicotinamide in urine. Men of all three groups show about the same levels of total plasma protein, hemoglobin, serum ascorbic acid and carotene, and urinary riboflavin. Even in the items where significant differences occur, the overlap of ranges between groups is so great as to raise doubt whether any of the associations is other than accidental.

A parallel analysis, in which the men were divided into groups with normal gingival tissues, with gingivitis only, and with gingivitis involving pocket formation, and their nutritive status contrasted by age groups, yielded essentially the same findings. Differences in serum protein, hemoglobin, vitamin A, vitamin C, and urinary riboflavin and *N'*methylnicotinamide were small, inconsistent, and not statistically significant. Differences in urinary thiamine were significant in each of the three age groups, but highest thiamine excretion was observed in men with advanced disease, in men aged seventeen to twenty-four years, and in men with the least disease in ages over twenty-five.

Additional data on patterns of periodontal disease in these groups and other oral findings are summarized in Table 3. The prevalence of disease was significantly lower in group A; groups B and C did not differ in this regard. Simple differences in prevalence did not fully account for differences in other findings. Pocket formation was observed in one man in four or five with disease in group A, about one man in three with disease in group B, and in nearly half the men with signs of disease in group C; mean PI scores limited to men with disease followed about the same pattern. These differences were somewhat less significant, statistically, than in full-group contrasts, principally because of the reduced numbers of men in the samples.

After the age of twenty-four years there were significantly fewer men in group A with obvious materia alba on two or more incisor teeth; men of groups B and C did not differ greatly in this respect. There was no difference between the groups in numbers of men with overt supragingival calculus on two or more teeth. Group A men over twenty-five years included fewer individuals with both materia alba and calculus, with non-significant differences appearing when groups B and C were compared. The prevalence of crowded arches was about the same in the three groups.

DISCUSSION

These findings are essentially negative; the original hypothesis—that a clear nutritional superiority would be found in men with the better periodontal conditions—is not supported.

These men were examined in March, when supplies of imported food are at the lowest point in the year and nutritional depletion most apt to be obvious if present at all. The most valid comparisons with nutritional status are probably those based on the periodontal score, which is a morbidity index and can reflect improvement as well as deterioration. In this population, however, about the same conclusions are drawn when comparisons are made with scores for mean gingival recession—an irreversible measure reflecting lifetime bone resorption status.

Individuals with marked depletion of one or another of the nutrients differed little, if at all, from their fellows not lacking in that nutrient. Eighten men, for example, were found with zero vitamin C levels in blood serum; their PI score, at their mean age of

TABLE 3

Additional Oral Findings in Three Groups of Alaska Scouts

Age Group (Years)	Group A	Group B	Group C	Approx. *P**
	Per Cent of Men with Signs of Disease			
17–24	45	71	62	0.04
25–34	57	86	80	<0.01
35 and over	41	82	95	<0.001
All ages	48	79	75	<0.001
	Per Cent of Men with Disease Showing Pocket Formation			
17–24	0	18	34	0.04
25–34	29	39	44	>0.20
35 and over	44	52	74	>0.20
All ages	23	35	45	0.03
	Mean PI Scores for Men with Disease			
17–24	0.60	0.71	0.95	0.10
25–34	0.73	1.28	1.53	0.07
35 and over	1.19	1.74	2.39	0.10
All ages	0.79	1.21	1.46	<0.001
	Per Cent with Materia Alba			
17–24	48	53	61	>0.20
25–34	27	64	75	<0.001
35 and over	50	80	90	<0.001
All ages	41	64	71	<0.001
	Per Cent with Supragingival Calculus			
17–24	39	43	33	0.20
25–34	60	71	73	0.20
35 and over	68	80	85	0.20
All ages	54	62	59	0.20
	Per Cent with Both Materia Alba and Supragingival Calculus			
17–24	19	31	24	0.20
25–34	20	49	63	0.001
35 and over	45	69	80	0.05
All ages	27	48	49	0.001

* Based on *F*-ratios for series of means, and on chi-square for proportions.

thirty-two years, was 1.26 ± 0.37, nearly identical with the score of 1.23 for all 128 men in the thirty to thirty-four age group. An additional 15 men showed less than 0.10 mg. of vitamin C per 100 ml. of serum; their mean PI score, at a mean age of 29.5 years, was 0.63 ± 0.20, rather lower than the whole-group score of 1.06 at this age point. Of these 33 men, 9 were free of overt gingivitis, and 12 showed one or more periodontal pockets, about the same proportions as those found for the whole group of men. Similarly, the 9 men deficient in serum vitamin A by ICNND standards—those with less than 10 μg. per 100 ml.—had a mean PI score of 1.00 ± 0.30, somewhat below that for the whole group at their mean age of 31.2 years, with gingivitis absent in two of the men and periodontal pockets present in three.

Subgroups A, B, and C follow no apparent geographic pattern and include about the same proportions of villages from the high-, the moderate-, and the low-caries groups analyzed in the previous report on dental caries in these men.[2]

Group A—men relatively free of past or present disease—included significantly fewer individuals in whom materia alba could be scraped from an incisor tooth with the explorer tine and significantly fewer in whom both materia alba and supragingival calculus could be observed. The apparent lack of association between calculus and disease shown in Table 3 permits only limited inference, since crevices were not probed; this evidence can support no statement beyond one that in this group men prone to heavy supragingival calculus formation seemed no more susceptible to disease than men without this tendency. Differences in oral cleanliness were not due to specific practices of oral hygiene or to differences in type or consistency of diet, so far as could be determined from a cursory comparison with reports from teams which examined civilians and their dietary practices in ten of the villages.

Of particular interest was the finding that men from about half the villages, including about 40 per cent of the men in the sample, tended to show active present gingival disease accompanied by no more than minimal indications of bone resorption. Their mean recession scores of 2.1, 11.2, and 18.1 in the three age groups are very little higher than scores of 2.3, 5.2, and 10.4 returned for Scouts considered to be free of gingival disease at the time of examination. At least two plausible hypotheses are suggested: that periodontal disease is a comparatively recent invader in these areas or that bone is relatively resistant to resorption in this group. Further study in these areas seems desirable.

SUMMARY

Six hundred and ninety-three men of the First and Second Scout Battalions of the Alaska National Guard were examined for periodontal disease in March, 1958. Determinations of total plasma protein, hemoglobin, serum ascorbic acid, vitamin A and carotene, and for urinary thiamin, riboflavin and *N'*methylnicotinamide were complete for 485 of the men. No association between signs of past or present periodontal disease and the levels of any of these nutrients could be demonstrated. Men from about half the villages represented showed very little indication of bone resorption, despite a high prevalence of active gingival disease.

REFERENCES

1. Russell, A. L., and Ayers, Polly. Periodontal Disease and Socioeconomic Status in Birmingham, Ala., *Am. J. Pub. Health*, **50**:206, 1960.
2. Russell, A. L., Consolazio, C. F., and White, C. L. Dental Caries and Nutrition in Eskimo Scouts of the Alaska National Guard, *J. D. Res.*, **40**:594, 1961.
3. Lantis, Margaret. *Alaskan Eskimo Ceremonialism*. New York: J. J. Augustin (American Ethnological Society), 1947.
4. Scott, E. M. Nutrition of Alaskan Eskimos, *Nutrition Rev.*, **14**:1, Jan. 1956.
5. Berry, F. B., and Schaefer, A. E. Program of the Interdepartmental Committee on Nutrition for National Defense, *J. Am. Med. Assoc.*, **166**:755, 1958.
6. Russell, A. L. A System of Classification and Scoring for Prevalence Surveys of Periodontal Disease, *J. D. Res.*, **35**:350, 1956.
7. Stahl, S. S., and Morris, A. L., Oral Health Conditions among Army Personnel at the Army Engineer Center, *J. Periodont.*, **26**:180, 1955.
8. Prevalence of Dental Conditions. In Commission on Chronic Illness, *Chronic Illness in a Large City*, Cambridge: Harvard University Press, pp. 107–18. 1957.

Relation of Protein, Carbohydrate, and Fat Intake to the Periodontal Syndrome

JAMES H. SHAW and DERRICK GRIFFITHS
Harvard School of Dental Medicine, Boston, Massachusetts

Earlier studies with the rice rat indicated that the complete replacement of carbohydrate in the periodontal syndrome-producing diet by fat and protein resulted in major reductions in the incidence and extent of the periodontal syndrome.[1] This influence was more striking in the soft tissues of the periodontium than in the alveolar bone. The present series of experiments was undertaken to examine this relationship more extensively and to determine the influence of major changes in the amount and type of carbohydrate, fat, and protein in the periodontal syndrome-producing diet.

EXPERIMENTAL METHODS AND RESULTS

Five experiments were conducted to determine the influence of variations in the carbohydrate, protein, and fat concentrations in diet 700 on the periodontal syndrome in rice rats. A sixth experiment was undertaken to determine whether the susceptibility to the periodontal syndrome varied with the age of the experimental subject. The 352 rice rats used in these six experiments were offspring of our breeding colony, which is maintained on Purina Laboratory Chow supplemented with small amounts of fresh lettuce and carrots three times a week.

In each experiment the subjects were distributed at weaning on the twenty-first day of age by littermates among the several groups. They were maintained singly for the appropriate experimental period in screen-bottom cages, with ration and tap water provided ad libitum. At the end of the experiment, the rice rats were sacrificed, and their heads preserved in 95 per cent alcohol in preparation for evaluation of the periodontal syndrome in the soft and in the calcified tissues by the method of Gupta and Shaw.[2] Experiments 1, 2, 3, and 6 were conducted about 2 years before experiments 4 and 5. During this interval a rigorous selection of breeders had been practiced in the breeding colony in an effort to increase the susceptibility of the offspring to the periodontal syndrome. At the same time, attempts were made to decrease the coefficient of variability within the colony as a whole and within litters in particular. As a result of these practices, it has been possible to reduce the assay period from 140 days in experiments 1, 3, and 6 and 155 days in experiment 2 to 84 days in experiments 4 and 5. These durations were determined as the most appropriate ones for the various experi-

This investigation was supported by U.S.P.H.S. research grant D-322 from the National Institute of Dental Research, National Institutes of Health.

Received for publication December 29, 1960.

mental populations on the basis of periodic in vivo oral examinations to determine the progression of the periodontal syndrome.

The designs of the first five experiments were similar. The first group of rice rats routinely served as controls to each other group and received the periodontal syndrome-producing diet 700. The composition of this diet and the various dietary modifications that were used in these experiments are presented in the upper half of Table 1. With the exception of diet 709, all replacements were made on an isocaloric basis, with the caloric values of 4, 4, and 9 being used for carbohydrate, protein, and fat. In the case of diet 709, a simple addition of 10 per cent corn oil was made to diet 705 in substitution for the usual 5 per cent level of vitaminized corn oil. The lower half of this table contains the experimental designs of these five experiments, with the group number placed under the diet which the rats in that group received throughout the experimental period.

The chief purpose of experiment 1 was to test the influence of the replacement of sucrose by dextrin. This replacement was tested in two comparisons: diet 705 involved a complete replacement of the sucrose in diet 700 by dextrin; diet 745 involved a complete replacement by dextrin of the sucrose in diet 740, where almost half the sucrose in 700 had been isocalorically replaced by lard.

Fifty-three rice rats were used in the four groups of the first experiment. The average rates of growth and the average final body weights for the animals in these four groups were excellent and almost indistinguishable from each other. The replacement of a little less than half the sucrose isocalorically by lard had no significant influence on the incidence and extent of the periodontal syndrome in either the soft or the calcified tissues of the periodontium. Likewise, the replacement of dextrin for sucrose in control diet 700 had no striking influence on the periodontal syndrome. The replacement of dextrin for sucrose in the diet with the reduced carbohydrate content led to a modest reduction in the periodontal syndrome in the soft tissues and a slightly smaller reduction in the syndrome in the calcified tissues. The latter reductions approached significance at the 5 per cent level.

Experiment 2 was designed to test the influence of replacement of sucrose by ground rice in two comparisons: the one a complete replacement of sucrose by rice ground to approximately the particle size of fine granulated sucrose and the other the replacement of sucrose by ground rice in diet 740, in which almost half the sucrose had been isocalorically replaced by lard. The influence of one-third replacement of sucrose by lard alone in diet 730 or with the replacement of an additional third of the sucrose by casein in diet 731 was also tested.

The five groups in the second experiment contained 62 rice rats. The data for this experiment are presented in Table 2. The diets used for all groups resulted in excellent growth and well-being. In both cases where ground rice was used in place of sucrose, striking reductions were observed in the number of soft-tissue areas involved by the periodontal score and in the scores for involvement of both the soft tissues and the calcified tissues of the periodontium. These reductions were large percentagewise but were either just barely significant at the 5 per cent level or approached significance at this level. Small reductions were observed in the number of areas involved by the periodontal syndrome in the calcified tissues, but these reductions did not approach statis-

TABLE 1

COMPOSITION OF DIETS IN GRAMS

Constituents	700	705	707	708	709	706	730	731	740	745	746	750	760	770	780	712	700A	712A
Sucrose	670	.....	170	330	.....	.....	490	290	400	.....	.....	310	130	0	0	790	670	790
Dextrin	.....	670	500	340	670	.....	.....	.....	.....	400	.....	.....	.....	.....	.....	.....	.....	.....
Rice	.....	.....	.....	.....	.....	670	.....	.....	.....	.....	400	.....	.....	.....	.....	.....	.....	.....
Vitaminized casein*	240	240	240	240	240	240	240	240	240	240	240	240	240	240	240	120‡	.....	.....
Casein	.....	.....	.....	.....	.....	.....	.....	200	.....	.....	.....	.....	.....	400	.....	.....	.....	.....
Egg albumin†	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	240	120‡
Lard	.....	.....	.....	.....	.....	.....	80	80	120	120	120	160	240	120	298	.....	.....	.....
Vitaminized corn oil*	50	50	50	50	.....	50	50	50	50	50	50	50	50	50	50	50	50	50
Corn oil	.....	.....	.....	.....	100	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Salts*	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Whole liver substance	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	Distribution of Experimental Groups among Diets by Group No.																	
Experiment No.:																		
1	1	2	.....	.....	.....	.....	.....	.....	3	4	.....	.....	.....	.....	.....	.....	.....	.....
2	1	.....	.....	.....	.....	2	3	4	.....	.....	5	.....	.....	.....	.....	.....	.....	.....
3	1	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	3	4	.....	5	.....	.....	.....
4	1	2	3	4	5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2	3	4
6	1–5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2–5	.....	.....	.....	.....

* *J. D. Res.*, 27:47, 1947.

† Vitaminized at same level as casein.

‡ Double portion of vitamins added to maintain same levels as in other diets.

TABLE 2

INFLUENCE OF SUCROSE REPLACEMENT BY RICE OR BY LARD AND CASEIN ON PERIODONTAL SYNDROME

Group No.	Diet No.	No. of Rice Rats	Soft-Tissue Lesions						Calcified-Tissue Lesions					
			No. of Areas			Extent of Areas			No. of Areas			Extent of Areas		
			Av.	S.E.M.*	C.R.†	Av.	S.E.M.*	C.R.†	Av.	S.E.M.*	C.R.†	Av.	S.E.M.*	C.R.†
Experiment 2:														
1.....	700	12	6.8	3.2	1.9	16.3+	8.4+	1.9	21.0	1.6	0.7	27.3+	8.4+	2.0
2.....	706	12	0.8	0.3	0.5	0.8+	0.3+	0.0	16.3	1.5	0.3	10.5+	1.1+	0.3
3.....	730	13	8.9	3.4	1.3	16.5+	6.7+	1.7	21.2	1.4	2.1	25.2+	6.0+	2.3
4.....	731	12	3.7	1.9	0.8	4.6+	2.5+	1.3	16.5	1.7	2.0	11.0+	1.5+	1.9
5.....	746	13	1.3	0.4	1.7	1.3+	0.4+	1.8	18.4	1.2	1.3	11.9+	0.9+	1.8
Experiment 3:														
1.....	700	11	14.4	3.8	0.8	22.3+	7.4+	0.1	19.5	2.4	0.9	31.1+	8.0+	0.3
2.....	730	12	10.1	3.7	1.7	21.2+	8.6+	1.8	16.3	2.5	1.7	27.7+	8.7+	1.9
3.....	750	14	6.5	2.6	2.0	7.1+	3.5+	2.3	14.0	2.2	2.0	14.3+	4.0+	2.4
4.....	760	13	5.2	2.8	2.4	4.4+	2.6+	2.7	13.6	1.7	3.6	11.1+	2.4+	3.0
5.....	780	14	4.5	1.9		2.3+	1.0+		10.6	0.8		7.3+	0.7+	

* S.E.M.—Standard error of mean.

† C.R.—Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when from 2.0 to 2.9, moderately significant; when 3.0 or higher, highly significant.

tical significance. Replacement of one-third of the sucrose by lard had no influence on the development of the periodontal syndrome, but when an additional third was replaced by casein, modest reductions occurred in the periodontal syndrome, especially with respect to the calcified tissues. The latter reductions were just barely significant at the 5 per cent level.

Experiment 3 was conducted to determine the influence of a stepwise replacement of sucrose isocalorically by lard, beginning with one-third of the sucrose in diet 730 increasing until all sucrose was replaced by lard in diet 780. The 64 rice rats in the third experiment were distributed into five groups. The data for this experiment are presented in Table 2. All groups grew well and were maintained in good health. Replacement of about one-third of the sucrose, as in diet 730, had no significant effect on the periodontal syndrome. Replacement of a little over half the sucrose by lard, as in diet 750, caused modest reductions in the periodontal syndrome that were just below significance at the 5 per cent level. Replacement of four-fifths of the sucrose by lard, as in diet 760, resulted in sizable reductions that were statistically significant at the 5 per cent level. Complete replacement of the sucrose by lard resulted in striking reductions in the periodontal syndrome that were statistically significant at the 5 per cent level for the soft tissues and at the 1 per cent level for the calcified tissues.

Experiment 4 was conducted to determine the influence of partial or total replacement of sucrose by dextrin and of a supplement of 10 per cent corn oil to the diet in which the sucrose had all been replaced by dextrin. Five groups, totaling 82 rice rats, were used. The replacement of one-fourth or half the sucrose by dextrin had no influence on the development of the periodontal syndrome. Complete replacement of sucrose by dextrin either with or without added corn oil led to modest decreases in the soft-tissue and calcified-tissue scores which were just significant at the 5 per cent level. The 10 per cent corn oil supplement gave no added benefit. Growth rates and final body weights were approximately identical for the five groups.

Experiment 5 was designed to test the influence of reducing the protein level of the diet from 24 to 12 per cent and of replacing casein by egg albumin at either 24 or 12 per cent. These manipulations had no influence on the periodontal syndrome. The averages for all criteria were almost identical among the four groups of 47 rice rats. In view of the negative nature of the data, they are not included in this paper. It is noteworthy that the rates of growth and the final body weights of the rice rats fed the diets containing 12 per cent of casein or egg albumin did not differ detectably from those of littermates fed the 24 per cent levels of protein. Growth rates were not penalized appreciably by the lower levels of protein.

Experiment 6 had a slightly different plan, based on the desire to determine whether susceptibility to the periodontal syndrome was dependent on the age of the experimental subject at the time the periodontal syndrome-initiating diet was begun. Diet 770, in which all sucrose had been isocalorically replaced by casein and lard, has been shown in earlier experiments to prevent practically completely the initiation of periodontal lesions in the soft tissues for at least 20 weeks.[1] Hence this diet was used for preliminary periods of varying lengths to permit the experimental subjects to reach specific ages with no more than a minor involvement of their periodontal tissues. Five groups with a total of 44 rice rats were used, of which the first group served as controls and were fed the periodontal syndrome-producing diet 700 immediately after weaning for 140

days. Groups 2 through 5 received diet 770 for preliminary periods after weaning of 21, 42, 63, and 84 days, respectively. At the end of these periods the rice rats in these four groups were transferred to diet 700, on which they were maintained for an additional 140 days. Thus the exposure of the rice rats in each of the five groups to ration 700 was identical in length but varied in respect to the initial age of the subjects. The average incidence of the periodontal syndrome in both the soft tissues and the calcified tissues was almost identical for the rice rats in all five groups. These data suggest that susceptibility to the periodontal syndrome did not vary detectably with age in this range. Further studies need to be conducted with different methods of preventing the onset of the periodontal syndrome.

DISCUSSION

The composite data from the first five experiments indicate the interrelated role of the collection of foodstuffs around the necks of the teeth and of the influence of the microbial flora on these substrates for the production of the periodontal syndrome in rice rats. Under dietary conditions favorable to these two circumstances, the syndrome developed rapidly in the susceptible subjects but had little or no influence on the periodontal tissues of the more resistant ones. Variability in susceptibility to this syndrome continues to be an urgent problem in this type of investigation and necessitates fairly large groups and large differences between the means for the data to be statistically significant. The majority of the offspring in the present generations of the susceptible strain are sufficiently susceptible to demonstrate levels of periodontal disease within 12 weeks that can be readily scored by the available methods. However, the remaining rice rats are sufficiently resistant on diet 700 or ones of comparable ability to produce the periodontal syndrome that they will not only remain free of any evidence of the syndrome for the 12- or 20-week experimental period but may remain so for a year or longer. Obviously, strong and undefined constitutional factors influence whether or not the periodontal syndrome will develop when the appropriate diets are fed. In each generation, rigorous standards are being followed in the selection of offspring in both susceptible and resistant strains, in order to reduce the variability within each strain.

The replacement of half or less of the sucrose in the control diet by lard had no detectable influence on the initiation and progression of the lesions of the periodontium. However, isocaloric replacement of more than half the sucrose by lard caused major reductions in the periodontal syndrome. When all sucrose was replaced by lard or a combination of lard and casein, the reductions produced were very striking. From the standpoint of percentage reduction, the influence was most pronounced in the soft tissues of the periodontium. Although the reductions in lesions of the alveolar bone were somewhat less striking percentagewise, these reductions tended to be somewhat more consistent in their extent. The high-fat, carbohydrate-low, or carbohydrate-free diets did not appear to permit the collection of the usual hard adherent masses around the necks of the teeth. Without these accumulations, little or no pathologic involvement of the soft tissues occurred, although there was a tendency for a slow but steady resorption of alveolar bone even in the absence of soft-tissue lesions.

The influence of the diets with partial or complete replacement of sucrose by dextrin was unimpressive, compared with the effect of comparable degrees of isocaloric replacement by lard. The dextrin-containing diets appeared to promote the collection of hard

Natural or Derived Materials Which Replace Ascitic Fluid for the Growth of an Oral Spirochete

T. A. NEVIN and H. V. JORDAN*

National Institute of Dental Research, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Maryland

Customarily, either ascitic fluid (Hampp, *J.A.D.A.*, **30**:1066, 1943) or blood serum (Noguchi, *J. Exptl. Med.*, **15**:81, 1912) has been added to infusion media such as that described by Hampp, Scott, and Wyckoff (*J. Bacteriol.*, **56**:755, 1948) for the cultivation of spirochetes; however, the nature of the growth factor supplied in these materials is not clear. Several substances were tested, therefore, and, upon obtaining evidence of activity, efforts were made to isolate the active component.

The test medium used was described by Nevin and Hampp (*J. Bacteriol.*, **78**:263, 1959) and is composed of Casamino acids, known growth factors, glucose, and minerals. The addition of a small amount of ascitic fluid to this medium is ordinarily required to support the growth of the test organism, *Borrelia vincentii* (strain N9). This system is easily reproducible. The following served as replacements for the ascitic fluid requirement described: heat-stable (100° C., 10 min.) acetone insoluble (4 vols.), pH 4.5 soluble fractions of either milk whey, veal heart infusion or canine ascitic fluid, restored to original volume with water and sterilized by filtration through sintered glass.

About 6×10^7 cells/ml were obtained routinely after 10 days' incubation at 37° C., when 0.05 ml. of ascitic fluid was added to 15 ml. of the test medium containing an inoculum of 1×10^6 cells. Growth appeared evenly dispersed throughout the medium. The acetone fractions described yielded fairly smooth growth also, but the addition of 0.5 ml. of the ascitic fluid fraction or 1.0 ml. of either the milk whey fraction or the veal heart infusion fraction was required for cell crops comparable to those obtained with whole ascitic fluid. Direct cell counts were made with a Petroff-Hausser chamber.

Two other substances which supported spirochetal growth were not fractionated. These were crystalline bovine albumin and mouse ascitic fluid.

Crystalline bovine albumin (Oyama, Steinman, and Eagle, *J. Bacteriol.*, **65**:609, 1953) yielded about 4.5×10^7 cells/ml when 0.1 ml. of a 1 per cent solution was used. Growth tended to be agglomerated rather than smoothly dispersed, however.

Mouse ascitic fluid, which was obtained by a method similar to that described by Lieberman, Douglas, and Humphrey (*Science*, **129**:775, 1959), proved comparable to the canine ascitic fluid used regularly. In the present work, a coagulase-negative micrococcus isolated from saliva was heat-killed (60° C., 30 min.), then suspended in Freund's incomplete adjuvant (Difco). Repeated intraperitoneal injections produced ascites within 18–24 days, permitting the collection of 1–3 ml. of fluid per mouse.

Qualitative studies of the acetone fractions, including the biuret, ninhydrin, Millon, and lead acetate tests, indicate that the active factor is a peptide containing tyrosine and a sulfur-containing moiety, probably cystine, as integral parts of the chain.

Received for publication September 6, 1960.

* Present address: Germfree Life Research Center, Tampa, Florida.

Toxicity Study of Stannous Fluoride in Swiss Strain Mice

V. A. SEGRETO, R. A. YEARY, R. BROOKS, and N. O. HARRIS

School of Aviation Medicine, Brooks AFB, Texas

Ninety mice were randomly allotted to six experimental groups (15 mice per group). Prior to experimentation each mouse was weighed, and this weight (av. wt. 31.5 gm.) was recorded. Rodents of each group received 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mg. of SnF_2, respectively. The stannous fluoride was administered as a 10 per cent solution via stomach tube under ether anesthesia. A few animals were discarded because of lung contamination caused by trachea penetration.

For an average weight of 31.5 gm., an LD-50/24 hours of 3.54 mg., was estimated by probit analysis, with a standard deviation of 0.42; 95 per cent confidence limits were therefore 2.70–4.38 mg. Since the LD-50 is presumably dependent on the weight of the animals, the weight was considered as a covariate in a second probit analysis. From this analysis, an LD-50/24 hours of 3.3 mg. was calculated, with a standard deviation of 0.2 mg. This established 95 per cent confidence limits of 2.9–3.7 mg.

The two estimates of LD-50/24 hours are not very different; however, the standard deviation of the estimate, when ignoring weight differences, is twice as large as the standard deviation using weight as a covariate.

There were no changes observed in the autopsy and histologic examinations which could be considered pathognomonic. The only consistent change which appeared correlated with dosage was the generalized *passive hyperemia,* apparent both grossly and microscopically. A limited incidence of focal–intra-alveolar hemorrhage was noted, but this could not be correlated with dosage. This condition occurred to a small extent in the control animals.

Histologic sections from one animal receiving 4.0 mg. demonstrated ulceration of the gastrointestinal tract. The liver of the same animal demonstrated periportal inflammation and small granulomas. These were isolated findings not occurring in any other animal.

The kidneys showed marked hyperemia but no tubular changes such as have been described in chronic poisoning with sodium fluoride. This is not surprising, since it has been reported that no histologic changes occur until after 28 days on a diet of 0.05 per cent sodium fluoride (J. J. Pendborg, *Acta pharmacol. et toxicol.*, **13**:36, 1957).

The spleens of all the animals contained megakaryocytes. The brains and hearts showed no microscopic abnormality.

It is interesting to note that there were no dramatic clinical manifestations with either the surviving or the expired animals. None of them went into complete collapse or coma. The cause of death in these animals was not apparent from the anatomic examination performed.

It should be emphasized that these mouse data cannot be extrapolated to apply to human toxicity because of species differences.

The authors are grateful for the statistical support of Richard C. McNee, Biometrics Branch, School of Aviation Medicine, USAF.

Received for publication October 1, 1960.

Cytology and Morphogenesis of Entamoeba Gingivalis

WAYNE W. WANTLAND, EDNA M. WANTLAND, and JOHN W. REMO

Illinois Wesleyan University, Bloomington, Illinois

Microscopic study of stages in the life-cycle of *Entamoeba gingivalis* from original samples and cultures and the employment of cytochemical methods have shed further light on the origin, development, form, and composition of such organelle structures as nuclei, karyosome, chromosomes, glycogen, and chromatoid bodies.

Stages found in mouth biomes and cultures with low floral populations were preponderantly immature cysts (2–4 nuclei) and mature cysts (8 nuclei). In iron-hematoxylin preparations (E. C. Faust and P. F. Russell, *Clinical Parasitology* [6th ed.; Philadelphia: Lea & Febiger, 1957], p. 947) mitotic figures in binary fission of trophozoites and multiple endogenous fission in cysts were similar, and in late metaphase and early anaphase 6 chromosomes were indicated, supporting Child's claim (*Univ. Calif. Publ. Zoöl.*, **28**:251, 1926) and refuting that of Stabler of 5 chromosomes (*J. Morphol.*, **66**:357, 1940) in this protozoan. Following excystation, division of octonucleated amoebas gives rise to 16 amoebulae. Nuclear reconstruction shows (1) the establishment of a thin nuclear membrane with a heavy, irregular layer of chromatin adhering to its inner surface; (2) a contraction of nucleoplasm, resulting in the formation of a multiform (3–4 parts), centrally located karyosome surrounded by a sparsely granular band extending about halfway to the nuclear membrane; and (3) the formation of 5 faint radiating lines resembling cartwheel spokes extending from the karyosome through both the granular band and a surrounding halo of clear nucleoplasm to the peripheral chromatin.

Study of nuclear structure in this amoeba supports the views of Kirby (*J. Parasitol.*, **31**:177, 1945), Kudo (*Protozoology* [Springfield, Ill.: Charles C Thomas, 1946], p. 351), and Chandler (*Introduction to Parasitology* [New York: John Wiley & Sons, 1955] p. 84) that the generic name *Entamoeba* instead of *Endamoeba* should be used for this protozoan on the basis of nuclear characteristics described in the type genus *Entamoeba hominis* tsd. *coli* by Casagrandi and Barbagalo (*Bol. Accad. Gioenia sci. nat. Catania*, N.S., fasc. 41, p. 7, 1895). The nuclear structure of *E. gingivalis* at no time during morphogenesis resembles that of amoebas typified by the cockroach colon parasite *Endamoeba blattae* (Bütschli), in which the comparatively large nucleus possesses a relatively thick membrane surrounding, from periphery to center, first, a fine chromatin network; second, from 15 to 20 spherical endosomes (karyosomes) arranged in a circle; and, lastly, a central coarse, reticulated area.

No contractile vacuoles have thus far been observed in the cytosome of either living or stained mouth amoebas. The cytoplasm presents a varied picture: at one time appearing granular and at another alveolar. In living trophozoites the cytoplasm usually appears granular, and, upon the formation of pseudopodia (mostly lobopodia with occasional filopodia), a clear hyaline area first appears in the region where the pseudopodium is to form, followed by a rapid streaming of granules in the direction of this temporary structure as it is rather explosively formed. In both trophozoites and cysts stained with iron-hematoxylin the cytoplasm appears predominantly granular, with a few darkly stained metachromatic bars and granules. Mitochondria, located as a rule near nuclei, are occasionally discernible in specimens stained with Janus green B or Janus red.

Young cystic stages stained with iron-hematoxylin showed a blunt, sausage-shaped cytosomal chromatoid body. Lugols (Grams iodine) solution (R. D. Lillie, *Histopathologic Technic and Practical Histochemistry* [New York: McGraw-Hill, 1957], p. 92), Donaldson's iodine-eosin reaction (*Lancet*, **1**:571, 1917), and periodic acid–Schiff reaction (H. A. Davenport, *Histological and Histochemical Technics* [Philadelphia: W. B. Saunders, 1960], p. 373) revealed from 1 to 2 large, irregularly shaped polysaccharide (glycogen) masses in immature cysts. That both chromatoid and glycogen bodies tended to disappear as cysts matured suggests a nutritive function.

Schiff's leucofuchsin, "cold Schiff" reagent (Lillie, *op. cit.*, p. 156), used in the nucleal reaction of Feulgen and Rossenbeck (*Z. physiol. Chem.* [Hoppe-Seyler], **135**:203, 1924), revealed absence of DNA in the cytoplasm of *E. gingivalis* trophozoites and cysts but a relatively high concentration of this nucleic acid in the karyosome, peripheral nuclear chromatin, and the cartwheel spokes of nuclei.

This study was supported by U.S.P.H.S. research grant D-357 from the National Institute of Dental Research, National Institutes of Health.

Received for publication November 30, 1960.

The Nasal Septum: An Endochondral Growth Center

LOUIS J. BAUME

Institute of Dental Medicine, University of Geneva, Switzerland

In an attempt to establish the centers of endochondral activity, operative in cephalofacial development, the heads of 12 human embryos ranging from 25 to 175 mm. were prepared for histologic analysis. In view of the impossibility of obtaining immediately fixed children's material, 6 heads of female *Macaca mulatta* ranging from 24 to 42 months of age, corresponding to approximately 7–14 human years, were used for the same purpose.

With the one exception of the condylar cartilage, all centers of endochondral ossification were found to originate within the viscero- and neurocranial cartilage primordium. Mid-sagittal sections of a 17-week-old human fetus (150 mm.) revealed a coherent cartilaginous skeleton comprising the entire skull basis, including the nasal septum. Two centers of ossification were forming at the basisphenoidal portion and occipital portion, each giving rise to a so-called basicranial synchondrosis. At a later stage (18 weeks) the ethmoidal center and presphenoidal center originate.

In all monkeys studied, the primordial cartilage persisted only in the following three places: (1) the spheno-occipital synchondrosis, (2) the prespheno-basisphenoidal synchondrosis, and (3) the nasal septum.

At higher magnification it could be seen that in these three centers endochondral ossification was proceeding. Endochondral ossification activity of the septal cartilage at this postnatal period has, to our knowledge, never been described. In the ethmoideoseptal synchondrosis, ossification contributes to the growth of the perpendicular plate of the ethmoid bone, whose body is also a derivative of the original unit of the basicranial cartilage primordium.

At the vomero-septal junction, which constitutes a syndesmosis between the cartilage septum and the membrane bone vomer, a picture unique in bone histology is seen. New cartilage is formed in the perichondrium without its being ossified enchondrally; the vomeral periosteum seems to be at rest.

Correlated processes of resorption at the nasal surfaces of the maxillary and palatine bones, as well as apposition at their palatal surfaces, evidence a growth movement exerted by the nasoseptal center in a vertical direction. Incremental lines at the cerebral surface of the ethmoid bone indicate corresponding upward movement of this bone. Thus neither De Coster's ethmoidal base line nor Korkhaus' palatal line used as reference in roentgenocephalometry can be considerated stable, although their peripheral outlines, as determined by the neighboring organs, may remain unchanged.

Histologic evidence is gained that the nasal septum constitutes a center of endochondral ossification, which conducts the vertical development of the middle and upper face; it remains active into a late postnatal period. Recognition of this growth activity should be helpful in the better understanding of the mechanism of facial development and management of certain cleft palate cases.

This study is supported by a research grant of the Swiss Odontostomatological Society.

Received for publication January 25, 1961.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Organized: New York City, December 10, 1920. Of those at the meeting of Organization, the following remain active members: Adolph Berger, Theodor Blum, A. H. Merritt, B. B. Palmer, L. M. Waugh.

Objects (*Const., Art. II*): The association has been established to promote broadly the advancement of research in all branches of dental science and in the related phases of the sciences that contribute directly to the development of oral health service, and as a whole; and, further, to encourage and facilitate co-operative effort and achievement by, and mutual helpfulness among, investigators in all nations in every division of stomatology to the end that dentistry may render cumulatively more perfect service to humanity.

Membership: Total (March, 1961), 1,097.

Eligibility.—Any person who conforms to the recognized standards of professional ethics and whose interest in dental science and dental research is shown by (*a*) research in process or conducted in the past, (*b*) scientific papers based upon original research presented before national or international meetings, or (*c*) scientific papers based upon original research and published in scientific journals is eligible for nomination to membership of the I.A.D.R.

Election.—New members are elected only on nomination by members and only by vote at the general meetings of the Association. Membership in a section or group does not confer membership in the Association. Nomination blanks for membership in this association may be obtained from the Secretary-Treasurer.

Endowment fund of the I.A.D.R. (as of October, 1959): $2,313.00.

OFFICERS OF THE ASSOCIATION

President: JAMES A. ENGLISH, *University of Buffalo*
President-Elect: SEYMOUR J. KRESHOVER, *National Institutes of Health*
Vice-President: DAN Y. BURRILL, *Northwestern University*
Secretary-Treasurer: JOSEPH C. MUHLER, *Indiana University*
Editor: FRANK J. ORLAND, *University of Chicago*

DIVISIONS AND SECTIONS

Division	*Section*	*President or Chairman*	*Secretary*	*Councilor*
North American	Ann Arbor	G. T. Chabeneau	E. R. Costich	J. K. Avery
	Baltimore	D. E. Shay	L. W. Gruber	E. C. Dobbs
	Birmingham	L. H. Schneyer	C. E. Klapper	S. B. Finn
	Boston	R. L. Glass	P. Goldhaber	H. H. Chauncey
	Chicago	J. R. Jarabak	A. A. Dahlberg	L. Fosdick
	Cleveland	S. W. Chase	M. M. Matt	P. E. Boyle
	Columbus	P. R. Weisenstein	S. Kolas	D. Permar
	Houston	B. Levy	D. A. Hagerman	S. S. Arnim
	Indianapolis	S. M. Standish	M. L. Swartz	W. G. Shafer
	Iowa City	W. Kern	D. E. Waite	A. K. Fisher
	Kansas City	B. K. Forscher	B. W. Warner	G. D. Roth
	Minnesota	A. Hampel	R. Stallard	L. Singer
	New York	H. J. Bartelstone	E. D. Goldsmith	G. Bevelander
	Pacific Northwest	B. S. Krause	B. J. Zeldow	L. Sreebny
	Philadelphia	H. Brenman	A. B. Hattler	N. B. Williams
	Pittsburgh	S. W. Leung	F. Draus	G. Cox
	Richmond	H. M. Syrop	G. W. Burke, Jr.	J. J. Salley
	Rochester, N.Y.	R. Lobene	H. A. Zander	E. Johansen
	St. Louis	J. W. Bynum	D. E. Flieder	J. R. Ring
	San Francisco		S. Epstein	W. C. Fleming
	Southern California	R. C. Grevlich	W. W. Wainwright	R. F. Sognnaes
	Toronto	D. L. Anderson	E. Harvold	G. Nikiforuk
	Washington, D.C.	R. M. Stephan	A. F. Forziati	W. C. Hess
	Wisconsin	C. W. Fairhurst	R. V. Winders	G. Ryge
British		M. A. Rushton	C. H. Tonge	A. I. Darling
Scandinavian	Denmark	P. O. Pedersen	J. J. Pindborg	J. J. Holst
	Norway	G. Toverud	I. Reichborn-Kjennerud	
	Helsinki	E. Tammisalo	K. Koski	
Japanese		S. Matsumiya	K. Enoki	G. Minoguchi
Not belonging to a Division	Johannesburg			A. J. Clement
	Mexico		F. R. Leycegui	
	Israel		S. Lewin-Epstein	
	Prague		F. Neumann	
	Austria, Vienna Center		E. Zitka	
	Australia	D. A. Cameron	G. S. Molyneux	A. R. Docking
Dental Materials Group		D. F. Taylor	M. L. Swartz	G. F. Paffenbarger

COUNCILORS-AT-LARGE

Clifton O. Dummett — Josse de Wever

*Supporting Associates, 1960–61**

***OF THE INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH AND THE* JOURNAL OF DENTAL RESEARCH**

THE ACRALITE COMPANY, INC., New York, N.Y.
JULIUS ADERER, INC., New York, N.Y.
AMALGAMATED DENTAL COMPANY, LTD., London
ASTRA PHARMACEUTICAL PRODUCTS, INC., Worcester, Mass.
BLOCK DRUG COMPANY, INC., Jersey City, N.J.
BRISTOL-MYERS INC., New York, N.Y.
L. D. CAULK COMPANY, Milford, Del.
COE LABORATORIES INC., Chicago, Ill.
COLGATE-PALMOLIVE COMPANY, Jersey City, N.J.
COLUMBIA DENTOFORM CORPORATION, New York, N.Y.
CRESCENT MANUFACTURING COMPANY, Chicago, Ill.
DENTISTS' SUPPLY COMPANY OF NEW YORK, York, Pa.
ENGELHARD INDUSTRIES, INC., Newark, N.J.
HANAU ENGINEERING COMPANY, INC., Buffalo, N.Y.
J. F. JELENKO & CO., INC., New York, N.Y.
H. D. JUSTI & SONS, INC., Philadelphia, Pa.
KERR MANUFACTURING COMPANY, Detroit, Mich.
ELI LILLY & CO., Indianapolis, Ind.
LORVIC CORPORATION, St. Louis, Mo.
MIDWEST DENTAL MFG. CO., Chicago, Ill.
THE J. M. NEY COMPANY, Hartford, Conn.
PRECIOUS METALS RESEARCH WORKS, INC., New York, N.Y.
THE PROCTER & GAMBLE COMPANY, Cincinnati, Ohio
RITTER COMPANY, INC., Rochester, N.Y.
ROCKY MOUNTAIN METAL PRODUCTS CO., Denver, Colo.
SCHERING CORPORATION, Bloomfield, N.Y.
SQUIBB INSTITUTE FOR MEDICAL RESEARCH, New Brunswick, N.J.
SURGIDENT, LTD., West Los Angeles, Calif.
S. S. WHITE DENTAL MFG. COMPANY, INC., Philadelphia, Pa.
THE UPJOHN CO., Kalamazoo, Mich.

ACADEMIC SUPPORTING ASSOCIATES

DENTAL MATERIALS GROUP, I.A.D.R.

* These Supporting Associates have made contributions in 1960–61 to the I.A.D.R. Treasury for support of the *Journal*. These contributions are in multiples of $100.